Effects of Chicken Egg Anti-F4 Antibodies and a Combination of Chitosan and Probiotic Supplementation on Performance and Diarrhea Incidences in Enterotoxigenic E*scherichia Coli K*88^{+} Challenged Piglets

by

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ABSTRACT

Post-weaning diarrhea is a major health challenge in the swine industry and is routinely managed by fortifying pig starter diets with antimicrobials. But there are concerns about antibiotic resistance, hence the need for identifying effective alternatives. The use of spray-dried whole egg powder containing anti-F4 antibodies (SDWE) against recombinant F4 antigens and chitosan oligosaccharide and *Enterococcus fecalis* probiotic combination (CPRO) was investigated in two trials using enterotoxigenic *Escherichia coli* $K88^+$ (ETEC) oral challenge model in 21-d-old piglets. Pre-challenge, SDWE supported higher ($P < 0.05$) piglet performance whereas during the post-challenge period, SDWE and CPRO had no effect on growth performance but diarrhea incidences and severity were reduced ($P > 0.05$) in SDWE-fed piglets compared to the control. The results show that SDWE supported greater piglet performance pre-ETEC challenge although there was no benefit of SDWE or CPRO supplementation evident during the post-challenge period in early-weaned pigs.

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FOREWARD

This thesis was prepared in a manuscript format. It contains two manuscripts corresponding to two chapters. Manuscripts one and two are being prepared for submission to the Canadian Journal of Animal Science and Animal Feed Science and Technology, respectively. Also, the two abstracts were accepted and presented as poster presentations at the 2015 ADSA-ASAS Joint Annual Meeting (JAM) Conference, Orlando, Florida, USA. The two manuscripts in this thesis were formatted to meet the Guidelines for Journal of Animal Science manuscript preparation.

DEDICATION

This work is dedicated to my beloved wife, Bosede Mary, my wonderful daughters, Temiloluwa Treasure and Opemipo Victoria, and to the memory of my late parents Chief Moses Adeleye Aluko and Mrs Janet Folorunso Aluko.

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LIST OF ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
AGPs	Antimicrobial growth promoters
BHI	Blood heart infusion
BW	Body weight
CCAC	Canadian Council on Animal Care
CD	Crypt depth
CEP	Control egg powder
CFU	Colony forming unit
COS	Chitosan oligosaccharide
CP	Crude protein
CPRO	COS + *Enterococcus fecalis* probiotic
CRD	Completely randomized design
d	Day(s)
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *E. coli*
Exp	Experiment
FBW	Final body weight
FC	Fecal consistency
G:F	Gain-feed ratio
GIT	Gastrointestinal tract
IBW	Initial body weight

IgY	Immunoglobulin in yolk
IL-6	Interleukin-6
ME	Metabolizable energy
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRO	*Enterococcus fecalis* probiotic
PUN	Plasma urea nitrogen
PWD	Post-weaning diarrhea
SAS	Statistical analysis
SDWE	Spray-dried whole egg containing anti-F4 antibodies
SDWE2	Spray-dried whole egg antibodies high inclusion rate (0.4%)
SDWE1	Spray-dried whole egg antibodies low inclusion rate (0.1%)
TNF-α	Tumor necrosis factor-alpha
VH:CD	Villus height to crypt depth ratio
VH	Villus height

CHAPTER ONE

GENERAL INTRODUCTION

In pig production, post-weaning diarrhea (PWD) is a major health problem with significant economic losses (Fairbrother et al., 2005; Daudelin et al., 2011) resulting from reductions in performance (Boudry et al., 2002, 2004), compromised intestinal health (Moeser et al., 2007), increased susceptibility to diseases and high mortality rate (Madec et al., 2000) during weaning transition in young pigs. Infection with enterotoxigenic *Escherichia coli* expressing the F4 ($K88^+$) fimbriae (ETEC F4) is one of the most important causes of PWD in pigs (Fairbrother et al., 2005). It has been shown that colonization of the small intestine of the pig by ETEC adhering to the epithelium accounts for most gastrointestinal disorders in both neonatal and post-weaning piglets (Yokoyama et al., 1992; Marquardt et al., 1999). Expression of an F4 (K88) fimbrial adhesin mediates bacterial attachment to specific F4 receptors located in the brush border of the swine intestine and secretion of enterotoxins resulting in diarrhea (Daudelin et al., 2011; Fairbrother et al., 2005). These enterotoxins which can be heat labile toxins (LT) or heat stable toxins (ST; subtypes STa and STb) activate cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) systems (Pluske et al., 2002). Consequently, there are increased secretions of sodium, chloride and hydrogen carbonate ions into the intestinal lumen and reduced absorption of liquid and salts leading to diarrhea, reduced feed intake and nutrient digestibility, retarded growth and even death (Heo et al., 2013). Overtime, this problem has been managed by in-feed sub-therapeutic administration of antimicrobial growth promoters (AGPs). For more than 50 years now, antimicrobials have been used in animal agriculture for growth promotion (sub-therapeutic doses), disease prevention (prophylactic doses) and treatment (therapeutic doses) [Diraviyam et al., 2014], and many reports have demonstrated the significant contributions of

antimicrobials to the improved performance of animals (Turner et al., 2001; Cromwell, 2002) and any replacement for AGPs would have to provide an improvement in performance that is economically viable (Dibner and Richards, 2005). However, due to increased public concerns about antimicrobial residues in animal products and the risk of the development of a reservoir of antibiotic resistant bacteria that cause diseases in human (Barton, 2000; Hulst et al., 2013; Diraviyam et al., 2014), much of the recent studies are focusing on identifying effective and viable alternative therapies (Owusu-Asiedu et al., 2003; Kiarie et al., 2009, 2011).

Therefore, among the strategies that have been proposed to improve growth performance and gut health in piglets include chicken egg antibodies (Wiedemann et al., 1990; Yokoyama et al., 1992; Marquardt et al., 1999; Owusu Asiedu et al., 2003; Kovac-Nolan and Mine, 2012), chitosan (Xu et al., 2014; Xiao et al., 2014) and probiotics (Delcenserie et al., 2008; Schlee et al., 2008; Daudelin et al., 2011). Passive immunization of piglets using chicken egg immunoglobulin (IgY) obtained from hyper-immunized laying hens possesses a variety of advantages over mammalian IgG such as convenience, high yield and cost effectiveness (Diraviyam et al., 2014). Various studies have demonstrated the protective and growth-promoting effects of egg specific antibodies in pigs (Yokoyama et al., 1992; Zuniga et al., 1997; Jin et al., 1998; Marquardt et al., 1999) whereas others did not find any significant effects (Chernysheva et al., 2004). In their study, Jin et al. (1998) demonstrated that purified antibodies against the fimbriae of *E. coli* $K88^+$ from chicken egg yolk were able to block the binding of *E. coli* $K88^+$ to the mucosal receptor thereby preventing the colonization of the small intestine and inhibiting enterotoxins production and expression by ETEC $K88^+$. Specific egg anti-F4 antibodies against ETEC $K88^+$ fimbrial antigens obtained from laying hens hyper-immunized with F4 fimbriae or antigens produced recombinantly

in a competent *E. coli* high expression system possibly would improve post-weaning pig performance and control PWD.

Also chitosan – a linear polysaccharide composed of randomly distributed beta (1-4) – linked D-glucosamine and N-acetyl-D-glucosamine has been shown in various studies to have inhibitory effects on *E. coli* in pigs (Haixiang et al., 2005), and to enhance gut health (Xu et al., 2014; Xiao et al., 2014), growth performance and nutrient digestibility in weaned pigs (Xu et al., 2014). The inhibitory effects of chitosan on Gram-negative bacteria such as *E. coli* have been attributed to its polycationic binding to the predominantly anionic cell surface of Gram-negative bacteria such as *E. coli* resulting in changes in outer membrane permeability and subsequent leakage of cell constituents such as enzymes and glucose thus, preventing its growth and spread and rendering *E. coli* more sensitive to the inhibitory action of bile and organic acids or ultimately, cell death (Helander et al., 2001; Rabea et al., 2003; Kong et al., 2010). On the other hand, probiotics are live microbial agents that have beneficial effects on the intestinal microbial balance of the host and are an effective factor to favorable health and functionality of the GIT. Various strains of bacteria have been used as probiotics and the most commonly used microorganisms include bacillus, yeast and lactic acid-producing bacteria such as *Lactobacillus*, *Streptococcus*, *Bifidobacterium* and *Enterococcus* (Stein and Kil, 2006; de Lange et al., 2010; Bednorz et al., 2013). The volatile fatty acids produced by lactic acid-producing probiotic bacteria possess potent bactericidal activity against members of *Enterobacteriacae* (Brocklehurst and Lund, 1990). Also they act competitively by exclusion in which attachment of probiotic microorganisms on the intestinal epithelial surfaces prevents pathogens such as *E. coli* from attaching (Stein and Kil, 2006).

It was hypothesized that supplementation of piglet diets with SDWE containing anti-F4 antibodies from laying hens immunized with recombinant F4 antigens and a combination of chitosan and *Enterococcus fecalis* probiotic (CPRO) will significantly improve growth performance and reduce ETEC-induced diarrhea in early-weaned pigs. Hence, the main objective of the present study was to determine performance and incidences of diarrhea in ETEC-challenged piglets when fed diets containing either chicken egg antibodies (SDWE) against recombinant F4 antigens or a combination of COS and *Enterococcus fecalis* probiotic.

CHAPTER TWO

LITERATURE REVIEW

Physiology of the gastrointestinal tract (GIT) of the pig

The GIT of a pig is a complex environment (de Lange et al., 2010) and there are changes in the gut particularly in newborns and during the weaning transition period such as in size, high protein turnover rates, microbiota development and establishment, and alterations in the digestive and immune functions (Pluske et al., 1997; Burrin and Stoll, 2003; Lalles et al., 2004). Functionally, the stomach receives and mixes feed, and is involved in partial digestion of feed and serves as a barrier against external environment (Barrow et al., 1977; Zhang and Xu, 2003). The stomach digestive function is mediated through the secretion of hydrochloric acid (HCl) that lowers the pH (Yen, 2000) for conversion of the gastric zymogens to active enzymes (Khan et al., 1999). Also the low pH values (3.0 to 4.0) of the stomach prevent passage of pathogenic microorganisms such as *E. coli* into the small intestine (Heo et al., 2013). During the weaning transition period, studies (e.g. Manners et al., 1976; Efird et al., 1982) have reported increased gastric pH values partly resulting from a lower acid secretion capacity of the stomach and reduced lactic acid production from lactose (Heo et al., 2013). This may be partly responsible for the susceptibility of early-weaned pigs to enteric pathogens including *E. coli* resulting in infections such as post weaning diarrhea (PWD).

After mixing and partial digestion of feed in the stomach, the chyme is then emptied into the small intestine for further digestion and absorption of nutrients. The emptying rate is controlled by gastric motility which has been shown to reduce in early-weaned pigs as compared to the suckling pigs (Snoeck et al., 2004). This reduction in stomach emptying rate can result to gastric

stasis and may contribute to the proliferation of enteric pathogenic bacteria and hence, development of PWD (Barrow et al., 1977).

Another important segment of the GIT is the small intestine which undergoes tremendous structural changes at the time of weaning (Hampson, 1986). The small intestine is lined with finger-like projections called villi (Zhang and Xu, 2003) and tubular glands that open into the intestinal lumen at the base of the villi known as crypts (Heo et al., 2013). Long villi are desirable for optimal function of the small intestine and they contain enterocytes at the brush-border surface. These enterocytes are responsible for releasing digestive enzymes and they account for approximately 95% of the epithelial cells (Cheng and Leblond, 1974). The crypts contain epithelial stem cells that are required for repopulation of epithelial cells (Llyod and Gabe, 2008) and 90% of the epithelial cells in the crypt are enterocytes involved in digestive activities via the release of digestive enzymes (Cheng and Leblond, 1974) which are mainly mucosa-based (Adeola and King, 2006). Various brush-border enzymes including, lactase, sucrase, maltase, glycoamylase (Kelly et al., 1991; Hedemann and Jensen, 2004), dipeptidylpeptidase IV, amino-peptidase N, alkaline phosphatase (Tang et al., 1999; Hedemann et al., 2003) have been investigated in weaned pigs and their activities used as indicators of maturation and digestive capacity of the small intestine (Henning, 1985; Hampson and Kidder, 1986). As part of the primary functions of the small intestine, crypt cells are also involved in fluid and electrolyte secretions and nutrient absorption from the intestinal lumen (Heo et al., 2013). This secretive function of the small intestine is a natural physiological phenomenon essential for nutrient digestion and absorption. But when there is imbalance between fluid and electrolyte secretion and absorption in the small intestine, pigs may be predisposed to secretory diarrhea (Pacha, 2000; Wapnir and Teichberg, 2002).

The large intestine consists of caecum, colon and rectum (Zhang and Xu 2003) and the mucosal surface is lined with crypts but lacking finger-like projections known as villi (Castillo et al., 2007). The physiological functions of the large intestine include fluid and electrolyte absorption, and provision of physical barrier against microbial invasion (Williams et al., 2001). Therefore, any changes in these functions may play a role in the pathogenesis of PWD and weaning has been shown to decrease the crypt density and increased mitotic index in the caecum of piglets (Castillo et al., 2007). Excessive fluid loss in the small intestine will result in PWD when absorption capacity of the large intestine is exceeded (Heo et al., 2013). Also, the large intestine harbours a large and diverse population of microorganisms that are involved in the digestion of carbohydrates and proteins that escape digestion in the small intestine (Pluske et al., 2002). The predominant bacteria in the large intestine include *Bifidobacterium, Clostridium, Fusobcterium, Bacteroides, Eubacterium* and *Propionibacterium* (Gaskins, 2001).

Effects of weaning on early-weaned piglets

The weaning transition is one of the most stressful events a young pig encounters in swine production (Xu et al., 2014). First, there is an abrupt interruption of the established social interaction and relationship with sow and littermates, then the stress of adapting to a new environment (Lalles et al., 2007). Second, sudden withdrawal of sow milk and adaptation to less digestible, plant-based dry diets containing complex protein and carbohydrate with various anti-nutritional factors (Cranwell, 1995; Lalles et al., 2007). Third, early-weaned piglets are removed from the passive protection they receive from sow milk making them to be more susceptible to enteric infections including ETEC infection (Yin et al., 2008). The sudden change in diet from sow milk to plant-based dry diet causes sharp reduction in feed intake immediately post-weaning (Pluske et al., 1997) in which approximately 50% of weaned piglets consume their first feed within

24-h and, 10% having weaning anorexia for up to 48-h (Brooks et al., 2001). Consequently, significant reductions in growth performance (Boudry et al., 2002, 2004; Heo et al., 2013), compromised intestinal health (Moeser et al., 2007), diarrhea (Heo et al., 2013) and high mortality rate (Madec et al., 2000) with significant economic losses (Fairbrother et al., 2005) are commonly observed.

Post-weaning diarrhea (PWD) in piglets

Post-weaning diarrhea (PWD) is a condition in weaned pigs characterized by frequent discharge of watery feces during the first two weeks after weaning (Cutler and Gardner, 1988). Post weaning diarrhea can be caused by a number of etiological agents including 1) bacteria: enterotoxigenic *E. coli* (ETEC), members of the genera *Clostridium*, *Lawsonia* and *Brachyspira* (Vondruskova et al., 2010), and 2) viruses: rotaviruses (Bertschinger, 1999; Thomsson et al., 2008), coronaviruses (Moller et al., 1998) and transmissible gastroenteritis virus (Madec et al., 2000; Thomsson et al., 2008). However, it has been variously demonstrated and recognized that diarrheal disease caused by ETEC is the most common enteric colibacillosis in neonatal and early-weaned pigs (Yokoyama et al., 1992; Alexander, 1994; Hampson, 1994) and an important cause of economic losses for pig producers (Amezcua et al., 2002). The clinical signs and death of pigs are caused by one or more toxins released by ETEC bacteria colonizing the small intestine (Zuniga et al., 1997). Among the different ETEC, those expressing the K88 (F4) fimbrial antigen are the most prevalent of *E. coli* infection found world-wide in pig production farms (Rapacz and Hasler-Rapacz, 1986) and has been estimated to be responsible for 50% of the piglet deaths each year (Marquardt et al., 1999).

ETEC strains responsible for PWD

Escherichia coli is a member of the autochthonous microbiota of pigs contributing to the maintenance of the microbial GIT balance (Gordon and Cowling, 2003) and are observed in both healthy and diseased pigs (Osek, 1999). In addition to commensal strains, pathogenic strains of *E. coli* responsible for either intestinal or extraintestinal diseases are a great health concern for both humans and animals (Bednorz et al., 2013). Various strains are classified into certain pathotypes according to possession of virulence-associated genes (VAGs) [Bednorz et al., 2013] including enterotoxigenic (ETEC), enteropathogenic (EPEC), Shiga-like toxin producing (STEC), uropathogenic (UPEC), newborn meningitis-causing (NMEC), septicemia-associated (SePEC) and avian-pathogenic (APEC) *E. coli* (Russo and Johnson, 2000; Kaper et al., 2004; Ewers et al., 2007). The intestinal *E. coli* of pigs are highly individual and dynamic (Katouli et al., 1995; Schierack et al., 2007) being influenced by diets, climate, age, and particularly weaning, which causes substantial changes in the intestinal microbiota (Franklin et al., 2002; Wu et al., 2007). Most frequently implicated ETEC strains (based on fimbrial or pilus antigens) as aetiological agents of PWD in pigs include F4 (K88), variants F4ab and F4ac (Nagy et al., 1990; Frydendahl, 2002; Harel et al., 1991) and F18, variants F18ab and F18ac (Imberechts et al., 1994; Rippinger et al., 1995).

Pathogenesis of post-weaning diarrhea (PWD)

The development of PWD and chain of events leading to diarrheal disease (pathogenesis) in pigs are not precisely clear as many diseases such as pneumonia can result to the same condition by compromising the immune function (Madec et al., 2000). Post weaning diarrhea has been associated with fecal shedding of large number of β-hemolytic ETEC that proliferate in the small intestine after weaning (Osek, 1999; Schierack et al., 2006) hence, usually called post weaning

colibacillosis (Fairbrother et al., 2005). Many types of bacterial pilus (fimbrial) adhesins may be involved in the attachment to the intestinal mucosa (Le Bouguenec, 2005) by attaching to glycoprotein receptors on the small intestine brush-borders of villous enterocytes, although how these interactions occur between fimbrae and receptors is not yet established (van den Broeck et al., 2000). These interactions result in the colonization of the GIT by ETEC and production and delivery of one or more enterotoxins such as heat labile toxins (LT) or heat stable toxins (ST; variants STa and STb) and subsequent activation of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) systems (Pluske et al., 2002). Consequently, there are increased secretions of sodium, chloride and hydrogen carbonate ions into the intestinal lumen and reduced absorption of liquid and salts leading to diarrhea, reduced feed intake and nutrient digestibility, retarded growth and even death (Heo et al., 2013). Because of lack of active immunity and damaged gut structural integrity, there is increased adhesion of pathogenic bacteria to the mucosal layer of the small intestine of affected piglets. Therefore, to minimize PWD through interventional strategies, for example, dietary interventions, such diets must possess the ability to either reduce the total number of pathogenic *E. coli* or prevent attachment to enterocytes or a combination of both characteristics (Heo et al., 2013).

Factors affecting PWD in piglets

Weaning transition period

Early separation of piglets from the sow denies them of the passive immunity derived from sow milk and contributes significantly to their susceptibility to enteric diseases (Bailey et al., 1992, 2005; Stokes et al., 2004). Because of immature intestinal immune system, there may be reduction in the ability to mount an appropriate immune response to pathogens or to tolerate dietary antigens (Heo et al., 2013). Weaning has been demonstrated to cause transient reduction in the ability of

intraepithelial lymphocytes to respond to mitogens (Bailey et al., 2005). Inflammatory responses are activated during weaning period with up-regulation of pro-inflammatory cytokines such as interleukin 1β, interleukin 6 and tumor necrosis factor-α (Pie et al., 2004). Also post-weaning anorexia coupled with its effects on gut morphology was reported to be a major contributing factor to the development of local intestinal inflammation during post-weaning transition period (McCracken et al., 1999).

Genetic

The relationship between ETEC colonization of the small intestine and presence or absence of appropriate brush-border receptors has been shown and emphasized to determine occurrence of diarrhea in neonatal and early-weaned piglets by various studies (e.g. Smith and Halls, 1968; Nagy and Fekete, 1999; Chernysheva et al., 2004). The interaction between *E. coli* pilus (fimbrial) adhesins and intestinal brush-border receptors causes the delivery of one or more enterotoxins with resultant secretory diarrhea (van den Broeck et al., 2000; Pluske et al., 2002). The increased susceptibility of individual piglets may be attributed to the presence of appropriate receptors for adhesins possessed by different *E. coli* strains (Stamm and Berstchinger, 1992). Hence, the genetic predisposition of pigs (possession of appropriate brush-border receptors) to colonization by certain *E. coli* strains is a contributing factor to the development of PWD as demonstrated by the above mentioned studies.

The GIT pH

When compared with sow-reared pigs, early-weaned pigs have higher gastric pH values partly due to a lower acid secretion capacity of the stomach at weaning coupled with reduced lactic acid production from lactose (Efird et al., 1982) and this may contribute to the susceptibility of

piglets to enteric infection (Heo et al., 2013). The intestinal pH values are a useful indicator of the gut health and microbial activity (Nyachoti et al., 2006) and at weaning, appropriate pH value is rarely maintained as a result of many factors such as diet changes and inability to produce sufficient HCl in the stomach and overeating after anorexia (Cranwell et al., 1995), lower secretion of saliva (Snoeck et al., 2004) and dietary electrolyte balance (Yen et al., 1981; Patience et al., 1987). Hence, less acidic intestinal pH values would provide an optimal conducive environment for colonization and proliferation of pathogenic microorganisms thereby predisposing piglets to PWD (Nagy and Fekete, 1999). On the other hand, a more acidic environment encourages proliferation and establishment of beneficial bacteria that have negative effects on pathogenic bacteria (Fuller, 1977). The intestinal pH values after weaning at different GIT segments have been reported to range from 5.4 to 7.4 by various studies (e.g. Pluske and Hampson, 2005; Nyachoti et al., 2006; Pierce et al., 2006; Wellock et al., 2006). In the large intestine, the pH values are dependent on diets that particularly contribute to the production of volatile fatty acids (VFAs) and lactate leading to the acidification of digesta. For example, resistant starch and fermentable fibres decrease pH values while branched-chain fatty acids and ammonia from protein fermentation increase pH values in the large intestine (Williams et al., 2001).

The gut microbiota

The GIT of piglets contains high number of various species of bacteria that are involved in the process of digestion (Vondruskova et al., 2010). Though born bacterium free, piglets quickly develop an established microbiota that is acquired from the feed and oral-fecal transmission in their post-birth environment (Heo et al., 2013). Microbial compositions are determined by 1) autogenic factors such as mutual interactions between the host and microorganisms, and also among different microorganisms (Jensen, 1998; Budino et al., 2005), and 2) allogenic factors such

as pH of the stomach, digestive enzymes, intestinal peristalsis, nutrients and immunity of the host (Budino et al., 2005; Roselli et al., 2005; Vondruskova et al., 2010). For gut health, immune system stability and well-being of piglets, the predominance of beneficial microorganisms over pathogens is essential (Makala et al., 2000; Mikkelsen et al., 2003). Shortly after birth (10 h to 12 h), the numbers of microorganisms can reach between 10^8 to 10^9 cfu/g feces and these numbers stabilize within 24 h to 48 h post-birth, although the microbial compositions are not definitive because of numerous changes occurring especially during weaning (Ducluzeau, 1983; Rojas and Conway, 1996; Roselli et al., 2005). In the stomach of a very young pig, *Lactobacillus spp*, *Streptococcus spp* and *Helicobacter spp* are dominant as they can tolerate the low pH environment (Jensen et al., 2001) with highest number of microorganisms being found in the caudal part of the intestines, where approximately 500 different species of microbes have been described and identified (Rojas and Conway, 1996; Budino et al., 2005). In a well-balanced microbial environment, members of the following genera prevail: *Lactobacillus, Bifidobacterium, Fusobacterium, Streptococcus, Enterococcus, Eubacterium, Peptostreptococcus, Enterobacter, Bacteroides* and *Porphyromona* while the numbers of coliform bacteria such as *E. coli* and *Clostridium spp* are lower (Fuller et al., 1978; Maxwell et al., 2004; Budino et al., 2005). Anaerobic conditions, favorable temperature, pH and slow passage of the digesta are the pre-conditions for the presence of large numbers of bacteria in the large intestine, up to 10^{10} cfu/g (Mikkelsen et al., 2003).

These bacteria positively influence their host through protection against pathogenic and conditionally pathogenic microorganisms in the form of colonization resistance (Jin et al., 1997; Roselli et al., 2005), competition for nutrients and binding sites, bacteriocin and short chain fatty acid (SCFA) production (Roselli et al., 2005), and subsequently, creation of immunological memory and development of intestinal immune system (Jin et al., 1997; Makala et al., 2000; Stokes

et al., 2004). But during weaning, a new microbial community is re-established such that milk-utilizing bacteria such as lactobacilli decrease markedly while potentially pathogenic bacteria such as coliforms increase (Heo et al., 2013). Hence, early-weaned piglets are more susceptible to proliferation and overgrowth of potentially pathogenic bacteria including ETEC (Hopwood and Hampson, 2003; Yin and Zheng, 2005).

Prevention and control of PWD

Following weaning, piglets are susceptible to enteric diseases such as PWD attributable to immature intestinal immune system, removal of immunoglobulin A (IgA) and other bioactive compounds derived from sow milk, increased gastric pH values and hence colonization and proliferation of pathogenic microorganisms including ETEC (Heo et al., 2013). The diarrheal disease caused by ETEC is the most common enteric colibacillosis in neonatal and early-weaned pigs (Yokoyama et al., 1992). The PWD coupled with reduced feed intake during weaning transition period has negative effects on growth performance in piglets and many strategies have been used in improving feed intake and health condition of weaned pigs. Prevention and control of PWD involves largely two approaches: 1) strategies that reduce predisposing factors to PWD, and 2) specific attempts to control pathogenic *E. coli.* Although antimicrobials have been used for growth promotion, disease prevention and treatment (Diraviyam et al., 2014), the swine industry is currently faced with identifying effective alternative approaches to antibiotic utilization that are not only cost effective but sustainable for pork production (Dibner and Richards, 2005) and also addressing public concerns about the risk of antimicrobial drug residues in food animals and antibiotic resistant bacteria. Therefore, in addition to antibiotics, various strategies such as the use of antibodies, chitosan, probiotics, prebiotics, acids, enzymes and other feed additives are being tried with different results.

Role of antimicrobial use in swine production

Antimicrobials represent an extremely important tool in the efficient production of pork and other animal products and when used at low (sub-therapeutic) levels in feeds, antibiotics improve growth rate and efficiency of feed utilization, decrease mortality and morbidity as well as improve reproductive performance while the intermediate (prophylactic) and high (therapeutic) levels are respectively used for disease prevention and treatment in animals (Cromwell, 2002). It has been estimated that 70 to 80% of pig starter and grower feeds, 50 to 60% of finisher and 40 to 50% of sow feeds contained antimicrobial agents including apramycin, chlortetracycline, lincomycin, neomycin, oxytetracycline, penicillin, tiamulin, tylosin, bacitracin zinc, sulfamethazine, arsenic acid and carbadox (Cromwell, 2002). The ability to suppress or inhibit the growth of certain microorganisms is a common property of these agents (Cromwell, 2002). As documented in the scientific literature (e.g. Hays, 1977; Zimmerman, 1986; Cromwell, 1991), thousands of experiments have demonstrated the efficacy of antibiotics in improving the rate and efficiency of growth as well as health status of pigs. For example, in studies involving young pigs, antibiotics improved growth rate by an average of 16.4% and feed utilization efficiency by 6.9% while in growing pigs, growth rate and feed efficiency were, respectively, improved by 10.6% and 4.5% (Cromwell, 2002). The mechanisms by which antimicrobial agents enhance growth performance and health condition of pigs and other food animals are not completely understood. Following early demonstrations (Coates et al., 1955; Coates et al., 1963) that oral antibiotics do not improve growth performance in germ-free animals, studies of the mechanism for growth promotion have been focused on interactions between the antibiotic and microbes (commensals and pathogens) and gut structural components (Dibner and Richards, 2005). First, antimicrobial growth promoters (AGPs) have been shown to decrease competition for nutrients and reduce

microbial metabolites that depress growth (Anderson et al., 1999). Second, enhanced nutrient digestibility as a result of the reduction in gut wall and villus lamina propria (Franti et al., 1972; Anderson et al., 1999). Also a reduction of opportunistic pathogens and subclinical infections, thereby allowing pigs to respond more closely to their genetic potential (Cromwell, 2002). Despite these growth and health benefits of antimicrobial utilization in food animal production, it is becoming inevitable that the use of AGPs will decline in the future particularly because of consumer pressure and export restrictions but it is unlikely that a single replacement for AGPs will be discovered to be economically viable (Dibner and Richards, 2005).

Concerns associated with antimicrobial growth promoters (AGPs)

In livestock industry, antimicrobial agents are broadly used for three purposes: treatment of sick animals (therapeutic), prevention of infections (prophylactic) and growth promotion (sub-therapeutic) [Landers et al., 2012]. Briefly, therapeutic use involves treatment of individual animals for a short period with the administration of antibiotic exceeding the minimal inhibitory concentration of known or suspected pathogen. Prophylactically, moderate to high doses of antibiotic are administered for a defined period to a group of animals while on the other hand, antibiotics used as growth promoters are administered over extended periods to entire herds or flocks (Barton, 2000). Antibiotic resistance is a looming public health crisis (Landers et al., 2012) and growth-promotant use is probably the area of highest concern because some of the antibiotics used are regarded as compromising the efficacy of some key human antibiotics and the duration of utilization may be for the whole life of the treated animals (Barton, 2000). Hence, there exists the potential threat to human health resulting from inappropriate antibiotic use in food animals as pathogenic-resistant micro-organisms propagated in these livestock are poised to enter the food supply and could be disseminated in food products (Garofalo et al., 2007). This led the European

Union (EU) to implement full ban on in-feed sub-therapeutic use of antibiotics in livestock diets in 2006 and there are increased pressure and interests to reduce or completely eliminate antibiotic use in food animal production in other parts of the world (Lusk et al., 2006; Heo et al., 2013). Consequently, a broad range of products as an alternative to antibiotics are being seriously focused on by the global biomedical research community (Diraviyam et al., 2014).

Promising alternatives to antibiotics in livestock agriculture

In-feed antibiotics have been consistently shown to improve pig weight gain and feed efficiency (Gaskins et al., 2002) but because of concerns attributed to antibiotic use in livestock production including antibiotic drug residues in food animals and antibiotic resistance development (Barton, 2000), a number of alternative strategies have been proposed and investigated (Heo et al., 2013) including chicken egg antibodies, chitosan, probiotics, prebiotics, acids, enzymes and other feed additives.

Chicken egg antibodies

Chicken eggs have been recognized sources of nutrients, including large quantities of egg yolk antibodies (Kovacs-Nolan and Mine, 2012) and the major components of yolk protein include apovitellenin, phosvitin, lipovitellin, apoproteins and livetins while the water-soluble fraction of yolk plasma consists of three types of lipoproteins known as α, β and γ-livetin (Liou et al., 2011). The γ-livetin (immunoglobulin in egg yolk, IgY), proved to be an IgG-like immunoglobulin, is functionally equal to the mammalian IgG. The IgY is transferred from the blood to the egg yolk and plays an important role in the passive protection of embryos (Polson and van Wechmar, 1980). Although oral administration of antibodies derived from serum and colostrum has been successful, it is prohibitively expensive to obtain large amounts of antibodies required (Kuhlman et al., 1988).

However, vaccination of laying hens provides a cheaper, convenient, high yielding and good source of antibodies (Yokoyama et al., 1992; Liou et al., 2011; Kovacs-Nolan and Mine, 2012). When compared to the production of antibodies from traditional immunization of rabbits, an immunized laying hen can yield antibodies equivalent to that from around 40 rabbits (Marquardt et al., 1999). The non-invasive method of chicken egg antibody production coupled with high egg production capacity holds a great promise for the therapeutic and prophylactic management of gastrointestinal disturbances in pigs and other food animals (Liou et al., 2011). For example, egg yolk antibodies from immunized laying hens had been applied for passive protection of neonatal and early-weaned piglets against ETEC-induced diarrhea (Yokoyama et al., 1992; Marquardt et al., 1999) and diarrhea caused by bovine rotavirus (Kuroki et al., 1994) or bovine coronavirus (Ikemori et al., 1997) in neonatal calves. After immunization of laying hens with ETEC pilus (fimbrial) antigens, eggs are collected and prepared for use in animals. The preparation can involve extracting antibodies from egg yolk, separating egg yolk from whole egg and or whole egg processed as powder either by freeze-drying (Wiedemann et al., 1990; Zuniga et al., 1997; Jin et al., 1998) or spray-drying (Yokoyama et al., 1992). Whole egg antibodies have been demonstrated to be considerably more resistant to gastric digestion and low pH than yolk alone and additionally, lysozyme, avidine and ovotransferrin components of egg white do have antibacterial properties against enterotoxigenic pathogens after oral administration (Wiedemann et al., 1990).

Chitosan

Chitosan, one of the most abundant natural polysaccharide biopolymers (Huang et al., 2007; Raafat et al., 2008), is a deacetylated chitin from the exoskeletons of arthropods such as crabs, shrimps and insects (Xu et al., 2013) and in the cell walls of fungi of the class *Zygomycetes* (Raafat et al., 2008). The global commercial production of chitosan is on a large scale (Singla and Chawla,

2001) and it has been estimated that up to 10^9 to 10^{10} tons of chitosan are annually produced in nature (Raafat et al., 2008). Chitosan consists of polymeric 1 → 4-linked 2-amino-2-deoxy-β-D-glucose, although preparations and batches vary with degree of deacetylation and polymerisation (Helander et al., 2001). Biodegradability, biocompatibility, nontoxicity and antimicrobial activities against fungi, bacteria and viruses are some of the unique biological properties of chitosan that have attracted much interests for its various applications (Dodane and Vilivalam, 1998). Each residue of chitosan contains amino and hydroxyl groups giving it many biological activities including haemostatic (Pusateri et al., 2006), anti-inflammatory (Dai et al., 2009), antitumor (Tsukada et al., 1990), antimicrobial (Limam et al., 2011; Benhabiles et al., 2012), hypoglycemic and hypocholesterolemic (Yao et al., 2006, 2008), immune-stimulatory (Moon et al., 2007) and growth-promoting (Xiao et al., 2014; Xu et al., 2014) properties. The poor solubility of chitosan makes it difficult for its use in food and bio-medicinal applications (Kim and Rajapakse, 2005). Unlike chitosan, its hydrolysed products such as chitosan oligosaccharides (COS) are readily soluble in water due to their shorter chain lengths and free amino groups in D-glucosamine units (Jeon et al., 2000). The use of chitosan in its oligosaccharide form (COS) by various researchers has been attributed to the low viscosity and greater solubility at neutral pH of COS (Kim and Rajapakse, 2005).

Although the exact mechanism of antimicrobial activity is unknown, interaction between positively charged chitosan molecule and negatively charged microbial cell membranes leads to outer membrane disruption, leakage of proteinaceous and other intracellular constituents (Rabea et al., 2003) indicating a damage to the bacterial cytoplasmic membrane (Chen and Cooper, 2002) and ultimately, the death of bacterial cells (Kong et al., 2010).

Probiotics

Probiotics are live microorganisms which when administered in adequate amounts confer health benefit on the host (FAO/WHO, 2002). A probiotic microorganism must have the ability to colonize the GIT, high growth rate and a low requirement for nutrients, suppress enteric pathogens, grow easily on a large scale under commercial conditions and survive in-feed and from the manufacturing process with stable activity (de Lange et al., 2010). Various strains of bacteria have been used as probiotics. The most commonly used species include lactic acid-producing bacteria of the genera *Lactobacillus, Streptococcus, Bifidobacterium* and *Enterococcus* (Bednorz et al., 2013). Probiotic microorganisms produce several inhibitory substances such as organic acids and hydrogen peroxide. These substances may limit the growth of harmful bacteria in the GIT. Also they act competitively by exclusion in which attachment of probiotic microorganisms on the intestinal epithelial surfaces prevents pathogens such as *E. coli* from attaching (Stein and Kil, 2006).

The genus *Enterococcus* is a member of the normal microflora of the GIT of pigs (Devries et al., 1994).Together with the other lactic acid-producing bacteria (LAB), i.e., lactobacilli and bifidobacteria, enterococci are widely used as probiotic products (Jin et al., 2000). *Enterococcus faecium* and *Enterococcus fecalis* are the most common species in the GI tract of humans and animals (Fisher and Phillips, 2009) and are used as starter cultures in food products such as cheese, probiotic cultures for humans and animals and as silage additives (Foulquie et al., 2006). They are micro-organisms capable of inhabiting environments with wide range of temperatures (10 to 45^{o}C), pH (4.6 to 9.6) and NaCl concentrations (up to 6.5%) [Carlos et al., 2010]. The volatile fatty acids e.g. lactic acid produced by these probiotic bacteria possess potent bactericidal activity against members of *Enterobacteriacae* (Brocklehurst and Lund, 1990). Furthermore, they have

been shown to inhibit bacterial adhesion to the small intestinal mucus (Jin et al., 2000) by either competing with pathogens for binding sites on the host cell surfaces or binding to the pathogen itself, blocking adhesive surface structures, resulting in reduced adhesive properties of the pathogen and hence decreased virulence (Bednorz et al., 2013). The gram-positive, facultative anaerobe and lactic acid bacterium *Enterococcus fecalis* has 'dualistic' behaviour toward human health (Franz et al., 2003; Foulquie et al., 2006; Christoffersen et al., 2012) as a multi-resistant, opportunistic pathogen (Linden and Miller, 1999; Fisher and Phillips, 2009) and a health-promoting probiotic in humans and animals (Domann et al., 2007; Nueno-Palop and Narbad, 2011; Han et al., 2013). This behaviour is strain-dependent as commensal enterococcal strains (developed and used as probiotics) differ from the clinical isolates possessing virulence factors (often responsible for nosocomial i.e. hospital-acquired, infections in humans) [Lempiainen et al., 2005; Abriouel et al., 2008]. Although results from studies are not consistent, dietary supplementation of probiotics has been reported to improve growth performance, villus height, and general intestinal health in broilers and pigs (Yeo and Kim, 1997; Smulikowska et al., 2005). Recently, Han et al. (2013) reported significant improvement in growth performance, health and diversity and composition of beneficial intestinal microorganisms in broilers fed diets containing microencapsulated *Enterococcus fecalis* probiotic compared to the basal diet fed group. In pigs, Mallo et al. (2010) reported a significantly improved growth performance and increased counts of beneficial flora (lactobacilli) in the piglet's intestine fed diet containing *Enterococcus faecium* probiotic. On the other hand, Scharek et al. (2005) concluded that supplementation of pregnant sow and piglet's feeds with *Enterococcus faecium* probiotic showed no obvious immune-stimulatory effect. However, it was found to have an impact on the microbial flora of the animals

and appeared to influence the early intestinal bacterial colonization of suckling piglets as reflected in the reduced enteropathogenic bacterial load.

Prebiotics

Prebiotics are dietary non-digestible food ingredients that alter the composition, or metabolism, of the gut microbiota in a beneficial manner (de Lange et al., 2010) and they have been referred to as the bifidus factor because they support the growth and/ or activities of probiotic microorganisms in the GIT (Gibson and Roberfroid, 1995; Rayes et al., 2009). The dietary supplementation of prebiotics influences volatile fatty acid content (VFA), branched-chain proportion, lactic acid and ammonia concentrations in the gut (Pie et al., 2007; Vondruskova et al., 2010). Short-chain fatty acids (SCFA) have been shown to stimulate natural bacterial activity, proliferation of bifidobacteria and other lactic acid-producing bacteria and as an energy source, particularly butyrate for enterocytes (Houdijk et al., 2002). The non-digestible food ingredients that are used as prebiotics include inulin, galacto-oligosaccharides, mannan-oligosaccharides, fructo-oligosaccharides, lactulose, isomalto-oligosaccharides, xylo-oligosaccharides and soybean-oligosaccharides (Grizard and Barhomeuf, 1999; Zimmermann et al., 2001; Tuohy et al., 2005). Their beneficial effects are thought to be mediated through selective stimulation of the proliferation and activities of bacteria associated with a healthy gut (Heo et al., 2013).

For example, bifidogenic effects of galacto-oligosaccharides, fructo-oligosaccharides and soybean-oligosaccharides have been reported to increase the numbers of *Bifidobacterium* and *Lactobacillus* genera (Roberfroid, 1998; Smiricky-Tjardes et al., 2003). The mannan-oligosaccharides modify microbial gut ecosystem by binding to the receptors present in the intestinal epithelium, thereby preventing the colonization of bacterial pathogens (Zimmermann et al., 2001) while lactulose is consumed by resident microflora in the large intestine to produce lactic

and/ or acetic acid, consequently stimulates the growth and activity of commensal bacteria especially of the genera *Bifidobacterium* and *Lactobacillus* (Gibson, 2004; Marinho et al., 2007). In similar manner, dietary supplementation with inulin has a positive effect on SCFA production, increased villus height, stimulation of commensal microflora and improvement of efficiency parameters (Crittenden and Playne, 1996).

Combination of prebiotics and probiotics (synbiotic) may increase the efficacy of probiotic effects on gut health and development in newly-weaned piglets (de Lange et al., 2010) by increasing the passage of probiotic bacteria through the upper part of the intestine and help the colonization of local receptors in the intestine (Roberfroid, 1998; Maxwell et al., 2004).

Diet acidifiers

At weaning, natural acidification of the stomach through hydrochloric acid (HCl) production is reduced due to the immature digestive system and sudden change in diet from milk to solid diets (Heo et al., 2013). In addition to the antimicrobial activity, organic acids and their salts have beneficial effect on digestibility, nutrient resorption (Roth and Kirchgessner, 1998) and performance of weaned and growing piglets (Kirchgessner et al., 1995; Partanen and Mroz, 1999; De Freitas et al., 2006). Furthermore, the positive effects of feeding diets containing acids to pigs can also be attributed to other factors such as: 1) lowering digesta pH, particularly in the stomach for protein digestion; 2) lowering stomach emptying rate; 3) providing nutrients for the intestinal tissue thereby enhancing mucosal integrity and function; and 4) stimulating enzyme production and activity in the small intestine (de Lange et al., 2010). According to their effects, organic acids can be classified into two groups: a) characterized by indirect effect in reducing bacterial populations by decreasing pH in the stomach e.g. lactic, fumaric and citric acids, and b) having direct effect of lower pH in the digestive tract on the gram-negative bacterial cell wall thereby

preventing their deoxyribonucleic acid (DNA) replication. This group includes formic, acetic, propionic and sorbic acids (Hansen et al., 1997; Castro et al., 2005).

Organic acids contribute to the stabilization of intestinal microflora because of their bactericidal properties and ability to improve enzymatic digestion (Kirchgessner et al., 1995; Marinho et al., 2007). As a result, the use of acids in piglets is essential because shortly after weaning, piglets suffer from a post-weaning syndrome associated with a typical starvation period (i.e. post-weaning anorexia). This is usually followed by ingestion of excessive amounts of feed, leading to mild acidification of stomach chyme and higher pH values contributing to the weakening of the protective stomach barrier function against bacterial pathogens such as *E. coli* and *Clostridium spp*. Consequently, pathogenic bacterial propagation and colonization in the small and large intestines (De Freitas et al., 2006; Vondruskova et al., 2010). In terms of impact on animal's physiology, lactic and butyric acids are of special interest (Mroz et al., 2006). The growth-promoting effects of lactic acid dietary supplementation in pigs have been documented and attributed to its anti-microbial activities and stimulation of endogenous enzyme production (Mroz et al., 2006). Meanwhile, butyric acid is a preferred energy source for enterocytes by enhancing intestinal cell proliferation (Kien et al., 2007). However, feeding butyric acid or relatively odorless sodium butyrate has little or undetectable effect on growth performance of newly-weaned piglets (Weber and Kerr, 2008; de Lange et al., 2010) and this may be partly attributed to endogenous fermentative butyric acid production (Sakata, 1987).

Feed enzymes

To improve the nutritive value of feedstuffs has been the major goal of using exogenous feed enzymes in swine diets achieved through the breakdown of anti-nutritional factors, elimination of nutrient encapsulation effect thereby increasing availability, breakdown of specific

chemical bonds in raw materials that cannot be cleaved by endogenous enzymes, and complementation of the enzymes produced by young animals (Bedford and Schulze, 1998; de Lange et al., 2010). In swine diets, most of the vegetable feedstuffs contain a considerable amount of non-starch polysaccharides (NSP) whose anti-nutritional effects are well-established and has been an area of intense research (de Lange et al., 2000). Studies have demonstrated improvement in the nutritional value of feedstuffs for young pigs with the use of appropriate enzyme preparations such as carbohydrase enzymes which eliminate the anti-nutritional activities associated with the NSP components of feed (Li et al., 1996; Omogbenigun et al., 2004; Meng et al., 2005). Enzymes may also improve performance of weaner piglets through the production of a variety of polysaccharide hydrolysis products that have direct effect on intestinal health by manipulating the growth of GI microorganisms (Williams et al., 2001; Pluske et al., 2002; de Lange et al., 2010). Also, feed enzymes may improve gut health by reducing the intestinal viscosity due to soluble NSP. This might decrease rate of digesta passage, diffusion of digestive enzymes and increase endogenous gut protein secretions thereby increasing substrate availability in the lower gut for microbial proliferation (Verstegen and Williams, 2002; Omogbenigun et al., 2004; de Lange et al., 2010).

Plant extracts and essential oils

Plant extracts and essential oils have been exploited in animal nutrition, particularly for their anti-microbial (Brambilla and De Filippis, 2005; Costa et al., 2007), anti-inflammatory, anti-oxidative (Liu et al., 2008), and anti-parasite properties (Magi et al., 2006). Essential oils have been used as artificial flavourings and preservatives in the manufacture of perfume, and over-the-counter formulations of medicines (de Lange et al., 2010). The anti-microbial activity of essential oils such as carvacrol and thymol is attributed to their delocalized electrons and the presence of a

hydroxyl group on the phenolic ring (Ultee et al., 2002) by initiating damage to the bacterial cell membrane. This compromises pH homeostasis and equilibrium of inorganic ions across bacterial cell membrane (Lambert et al., 2001) and subsequently, leading to the collapse of the proton motive force and depletion of the adenosine triphosphate (ATP) pool in the microbe (Ultee et al., 2002). To prevent diarrheal diseases, it is important to focus on the extracts and essential oils that inhibit the proliferation of *E. coli* which is a common cause of intestinal diseases in piglets (Vondruskova et al., 2010). For example, Khan et al. (2009) demonstrated that pathogenic strains of *E. coli* are sensitive to plant extracts such as *Acacia nilotica*, *Syzygium aromaticum* and *Cinnamum zeylanicum.* But a major disadvantage of plant extracts is that their compositions are unstable (Bomba et al., 2006) due to the influence of climate, season and harvesting methods (Borovan, 2004). These factors may probably have contributed to increased controversial and inconsistent results from different scientific studies investigating the effect of plant extracts in animal nutrition (Vondruskova et al., 2010). Plant extracts in pig nutrition include: oregano, cinnamon and Mexican pepper (Manzanilla et al., 2004), sangrovit from alkaloids of *Maceaya cordata* (Borovan, 2004), thyme, clove, eugenol and carvacrol (Oetting et al., 2006) and aged garlic extract, allicin (Tatara et al., 2008). These plant extracts and essential oils with their characteristic odour or flavour may improve performance in animals not only by controlling enteric pathogens but by increasing palatability of diets (de Lange et al., 2010).

CONCLUSIONS

The weaning transition period is regarded as one of the most stressful conditions being encountered by young pigs with attendant significant economic losses in swine production. These losses result from compromised intestinal health, increased susceptibility to diseases especially post-weaning diarrhea, retarded growth rate and high mortality rate. Diarrheal disease caused by enterotoxigenic *E. coli* (ETEC) is the most common enteric colibacillosis in neonatal and early-weaned piglets and ETEC expressing K88 (F4) fimbrial antigens has been estimated to be responsible for 50% of piglet deaths annually. Therefore, antimicrobial growth promoters (AGPs) are routinely included in the pig starter diet to prevent and control infectious diseases and improve growth performance.

However, there are concerns about the use of AGPs in livestock industry because of the risk of antimicrobial drug residues in food animals and the development of a reservoir of anti-microbial resistant bacteria that pose a serious and looming public health concern. Currently, the swine industry is facing the challenge of identifying means for controlling diarrhea and enhancing growth performance in young pigs coupled with the ban on AGP use in livestock production in European Union countries and the increasing pressure to follow same in other parts of the world. Hence, most recent studies are focusing on identifying a broad range or a combination of products as viable and effective alternatives to AGPs, although with inconsistent results and drawbacks. Some of the promising alternative means include chicken egg antibodies, polysaccharides such as chitosan, pre- and probiotics, feed enzymes, organic acids, plant extracts and essential oils and other additives.

The use of chicken egg specific antibodies from laying hens hyper-immunized with recombinantly produced specific bacterial antigens is one of the proposed promising effective and

economically viable alternative means of controlling diarrhea and enhancing growth performance in neonatal and early-weaned piglets. Vaccination of laying hens provides a cheaper, convenient, high yielding and good source of antibodies. Also, it's less invasive and requires small number of animals when compared to obtaining antibodies from mammalian sources such as rabbits. This supports the three Rs (replacement, refinement and reduction of number of animals) in the use of animals in experiments. Various studies have demonstrated that the oral administration of anti-F4 antibodies obtained from laying hens immunized with wild type F4 fimbrial antigens is highly effective in controlling ETEC-F4 pathogenesis. However, the efficacy of antibodies produced against recombinant F4 fimbrial antigens to be equally effective has not been demonstrated in a study with piglets.

Other strategy that can be exploited for the control of diarrheal diseases and improvement of growth rate in piglets is a combination of potential alternatives such as synergistic effects of chitosan oligosaccharide and *Enterococcus fecalis* probiotic. Being one of the most abundant polysaccharide biopolymers, chitosan combines a unique group of characteristics: biodegradability, biocompatibility, nontoxicity, antimicrobial activities against fungi, bacteria and viruses, and increased nutrient digestibility in chickens and pigs. The genus *Enterococcus* is a member of the beneficial microbiota of pigs and lactic acid-producing bacteria (LAB). *Enterococci* inhibit pathogenic bacterial colonization of the intestine and produce lactic acid which has potent antibacterial activity against pathogenic microorganisms such as *E. coli*. Therefore, to demonstrate the significance and advantages of chicken egg antibodies against recombinantly produced specific F4 antigens and a combination of chitosan and *Enterococcus fecalis* probiotic, critical investigations and evaluations are warranted.

CHAPTER THREE

MANUSCRIPT I

Effects of chicken egg anti-F4 antibodies supplementation on performance and diarrhea incidences in enterotoxigenic *Escherichia coli* K88^{+} challenged piglets

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ABSTRACT

This study was conducted to evaluate the effects of feeding diets supplemented with spay-dried whole egg containing anti-F4 antibodies (SDWE) against recombinantly produced F4 antigens to piglets challenged with enterotoxigenic *Escherichia coli* K88 (ETEC). Twenty-seven individually housed piglets [(Yorkshire x Landrace) x Duroc, 7.27 ± 0.47 kg initial BW] weaned at 21 ± 1 d were randomly allotted to 3 dietary treatments (n = 9) consisting of a wheat-soybean meal basal diet containing either 0 (control egg powder; CEP), 0.1% (SDWE1**)** or 0.4% (SDWE2) SDWE for a 14-d study. After a 7-d adaptation period, all pigs were weighed, blood samples collected and then orally challenged with 6 mL (2 x 10^9 cfu/mL) of freshly grown ETEC inoculum on d 8. Pigs had *ad libitum* access to feed and water throughout the study. Blood was sampled at 24 h and 48 h post-challenge to determine plasma urea nitrogen (PUN) content and diarrhea incidences and fecal consistency scores were recorded from d 9 to d 12. On d 14, all pigs were weighed and then euthanized to obtain intestinal tissue samples for villus height and crypt depth measurements. During the pre-challenge period, pigs fed the SDWE2 diet had higher ($P < 0.05$) average daily gain, ADG and gain to feed ratio, G:F compared to CEP but there were no differences among treatments in any of the performance response criteria during the post-challenge period. Incidences of diarrhea were similar among treatments although piglets fed SDWE-containing diets recovered from diarrhea within 48 h (with fecal consistency score of 0.0) of ETEC challenge compared to CEP pigs. Also, fecal shedding of ETEC, PUN content and intestinal histomorphology were similar among treatments. The results show that SDWE at 0.4% supported greater piglet performance before challenge although there was no significant benefit of SDWE supplementation at either 0.1% or 0.4% evident during the post-challenge period.

Keywords: ETEC $K88^+$, chicken egg anti-F4 antibodies, recombinant F4 antigens, pigs

INTRODUCTION

In pig production, post-weaning diarrhea (PWD) is a major health challenge with significant economic losses (Fairbrother et al., 2005; Daudelin et al., 2011) resulting from reduced growth performance (Boudry et al., 2002, 2004), compromised intestinal health (Moeser et al., 2007), increased susceptibility to diseases and high mortality rate (Madec et al., 2000). Infection with enterotoxigenic *Escherichia coli* expressing the $K88^+$ (F4) fimbriae (ETEC F4) is one of the most important causes of PWD in pigs (Fairbrother et al., 2005). It has been shown that colonization of the small intestine of the pig by ETEC adhering to the epithelium accounts for most gastrointestinal disorders in both neonatal and early-weaned piglets (Yokoyama et al., 1992; Marquardt et al., 1999). For more than 50 years, antimicrobials have been used in animal agriculture for growth promotion (sub-therapeutic doses), disease prevention (prophylactic doses) and treatment (therapeutic doses) [Diraviyam et al., 2014], and many reports have demonstrated the significant contributions of antimicrobials to the improved performance of animals (Turner et al., 2001; Cromwell, 2002). Fortifying starter diets with antimicrobial growth promoters (AGPs) is routinely used for controlling and mitigating effects of PWD in pigs (Pluske et al., 2002). However, there are public concerns about antimicrobial drug residues in food animals and the risk of the development of a reservoir of antibiotic resistant bacteria that cause diseases in human (Barton, 2000; Hulst et al., 2013; Diraviyam et al., 2014). Hence, much of the recent studies are focusing on identifying effective and viable alternative therapies (Owusu Asiedu et al., 2003a; Kiarie et al., 2009, 2011) and one of these promising alternative means is passive immunization of piglets using chicken egg antibodies against recombinant F4 antigens. To our knowledge, the efficacy of anti-F4 antibodies against ETEC $K88^+$ fimbrial antigens produced from laying hens hyper-immunized with recombinant F4 fimbrial antigens has not been investigated in pigs. After

the original isolation of F4 fimbrial antigens from the wild type strain of ETEC F4 for the immunization of laying hens, it is now produced recombinantly in a competent *E. coli* high expression system (Zyme Fast System Inc., Winnipeg, MB, Canada). Zyme Fast System Inc. demonstrated *in vitro* that its new antibodies produced against the recombinant F4 antigen had an identical ability to bind to both the wild type and the recombinant antigens, and that of the whole ETEC-F4 organism.

Therefore, the objective of this present study was to determine growth performance and incidences of diarrhea in ETEC $K88^{+}$-challenged piglets when fed diets containing chicken egg antibodies against recombinant F4 antigens.

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care Committee of the University of Manitoba. Pigs were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

Animals, treatments and oral challenge

A total of 27 piglets [(Yorkshire x Landrace) x Duroc, 7.27 ± 0.47 kg initial BW] weaned at 21 ± 1 d from the University of Manitoba's Glenlea swine research unit were used in this study. Pigs were individually housed in cages (dimensions: 2.5 Ft. x 2 Ft. x 15 in.) within a room at the T. K. Cheung Centre for Animal Science Research, University of Manitoba, Winnipeg, Manitoba, Canada. Piglets were randomly allotted to 3 dietary treatments (n = 9) consisting of a wheat-soybean meal basal diet containing either 0 (control egg powder; CEP), 0.1% (SDWE1**)** or 0.4% (SDWE2) SDWE for a 14-d study. Room temperature was maintained at 30 ± 1^{o}C and pigs had *ad libitum* access to feed and water throughout the experimental period. The basal diet (Table 1)

was formulated to meet the NRC (2012) nutrient specifications for 5 to 10 kg body weight pigs. SDWE containing anti-F4 antibodies (antibody titer of 8.1 x 10^5) against recombinant F4 fimbrial antigens and CEP were supplied by Zyme Fast System, Winnipeg, Manitoba, Canada. After the original isolation of F4 fimbrial antigens from wild type strain of ETEC F4 for the immunization of laying hens, it is now produced recombinantly in a competent *E. coli* high expression system (Zyme Fast System Inc., Winnipeg, MB, Canada). Procedures for the immunization of laying hens, harvesting and processing of eggs from immunized hens to obtain antibodies are as previously demonstrated (Yokoyama et al., 1992; Jin et al., 1998; Marquardt et al., 1999).

Body weights (BW) and feed intakes were determined weekly and average daily BW gain (ADG), average daily feed intake (ADFI) and the ratio of BW gain to feed intake (G:F, i.e., feed conversion efficiency, FCE) were calculated. After a 7-d adaption period, each piglet was bled (venipuncture through the jugular vein) to obtain blood samples and then orally challenged with 6 mL (2 x 10^9 cfu/mL) of freshly grown ETEC-F4 inoculum on d 8. Fecal samples were also collected before the ETEC-F4 challenge for enumeration of fecal shedding of ETEC. Pigs were monitored for another 7 d post challenge for incidences of diarrhea and general health conditions. Incidences and severity of diarrhea were assessed on a cage basis by two trained independent personnel (without prior knowledge of dietary treatment allotment) using a fecal consistency scoring system [0 = normal feces; 1 = soft feces; 2 = mild diarrhea; 3 = severe diarrhea (Marquardt et al., 1999)]. The fecal consistency for each piglet was determined by averaging the assigned scores by two independent personnel and a score of ≤ 1 was considered to indicate no diarrhea.

ETEC K88+ and culture condition

The ETEC-F4 strain was originally obtained from Veterinary Diagnostic Services of Manitoba, Winnipeg, Manitoba, Canada. To evaluate the proliferation of ETEC-F4 and to

differentiate the inoculum from the indigenous strains, the pure ETEC-F4 was made resistant to ciprofloxacin in Mueller-Hilton broth (Becton Dickinson and Company, Sparks, MD) as previously described by Opapeju et al. (2009). The suspension concentration of 10^9 cfu/mL was used for the oral challenge.

Table 1. Composition and calculated nutrient levels of basal diet (d 21 to d 35, as-fed basis)

Item	%
Ingredients	
Barley	17.00
Wheat HRW	27.00
Soybean meal	24.16
Fish meal	5.00
Dried whey	19.00
Vegetable oil	5.00
Limestone	0.50
Calcium monophosphate	0.40
Iodized salt	0.50
Vitamin-mineral premix[1]	1.00
Lys-HCl	0.30
DL- methionine	0.07
Threonine	0.07
Tryptophan	-
Total	100.00
Calculated nutrient levels	
ME, kcal/kg	3375
CP	22.46
Lys	1.46
Met	0.40
Met + Cys	0.70
Thr	0.82
Trp	0.25
Ca	0.89
Av.P	0.45

[1]Vitamin-premix provided per kg of diet: vitamin A 8,250 IU, vitamin D3 835 IU, vitamin E 40 IU, vitamin K3 4 mg, vitamin B12 0.025 mg, vitamin B1 2 mg, vitamin B2 12 mg, nicotinic acid 22.5 mg, folic acid 2 mg, pyridoxine 4.5 mg, biotin 0.2 mg, pantothenate 15 mg, choline 500 mg, Mn 50 mg, Fe 100 mg, I 0.4 mg, Cu 25 mg, Zn 150 mg, Se 0.3 mg.

Blood and fecal sample collections

Heparinized and non-heparinized blood samples to obtain plasma and serum, respectively, for plasma urea nitrogen (PUN) and serum cytokine concentration analyses were collected from the jugular vein of each piglet on d 8 (before *E. coli* oral inoculation), d 1, 2 and 7 (post *E. coli* challenge). Samples were centrifuged at 2,000 x g for 10 min at 5^{o}C to harvest plasma and stored at -20^{o}C until required for PUN analysis. Also, sera from non-heparinized blood samples were harvested and stored at -20^{o}C until required for serum cytokine (interleukin 6, IL-6 and tumor necrosis factor alpha, TNF-α) concentration analysis using ELISA (Quantikine ELISA, R&D Systems Inc., Minneapolis, USA) according to the manufacturer's instructions. Fecal samples (about 2 g) were collected from each piglets on d 8 (pre *E. coli* challenge), at 12 h (for PCR *E. coli* K88^{+} genotyping, results not shown), d 1, 2 and 7 post *E. coli* challenge and then stored at -80^{o}C until required for further analyses. Culture-based *E. coli* enumeration analysis was performed using 1 g of fecal sample from each piglet in 9 mL sterile 0.1 % peptone water, vortexed for 60 s and a 10-fold dilution was made in sterile peptone water. The ETEC K88^{+} in the serially diluted samples were quantified using Eosin Methyl Blue agar (Becton Dickson and Company) with ciprofloxacin (0.5 µg/mL). The plates were incubated aerobically at 37^{o}C for 24 to 48 h and then colonies were counted.

Digesta and intestinal tissue collections

Ileal sections and digesta were collected from each piglet on d 7 post ETEC challenge after being anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and euthanized by an intracardiac injection of sodium pentobarbital (50 mg/kg of body weight; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada). The digesta samples were collected from ileum 15 cm cranial to the ileocecal

junction in sterile sample bags, preserved in ice pack and transferred to -80^{o}C until required for ETEC K88^{+} enumeration analysis. The ileal tissue samples were preserved in 10% formalin to fix the villus and crypt for subsequent histomorphometric measurement.

Intestinal histomorphology

Cross-sections from formalin-fixed samples were processed for histological examination using the standard Hematoxylin and Eosin (H&E) method. Measurement of villus height (VH) and crypt depth (CD) was made on at least ten well-oriented villi per specimen using a Zeiss photomicroscope equipped with a Sony 3 chip CCD color camera (Carl Zeiss, Oberkochen, Germany). Captured images were analyzed using NIH ImageJ software (NIH Image, Bethesda, Maryland, USA) with the height of the villus being measured from the tip to the villus-crypt junction and the CD from this junction to the base. The VH from each piglet was obtained by averaging measurements of at least 10 well-oriented villi with corresponding crypt depths and the villus to crypt ratio (VH:CD) determined.

Statistical analysis

All data were subjected to statistical analysis using the Mixed Procedure of SAS, 9.4 version (SAS Institute Inc., Cary, NC, USA). Cage was the random effect and diets (main effects of CEP and SDWE) were the fixed effects. Bacterial enumeration data was transformed to $\log_{10}$ cfu/mL before statistical analysis. Means for significant treatment differences were compared by the least significant difference (LSD) test. Chi-square test was performed on diarrhea incidences to determine if differences among the treatment groups were significant. Probability values of $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The growth performance results are shown in Table 2. Final BW was similar among the three treatments ranging from 8.70 kg (CEP) to 9.18 (SDWE2) kg with piglets fed diet containing SDWE2 gaining 26.97% weight as compared with 19.18% in CEP fed piglets at the end of 14-d experimental period. During wk 1 (pre-challenge), the ADG was affected by diet ($P = 0.011$) with SDWE2-fed piglets having the highest (110.74 g/d) ADG while there was no statistically significant difference between SDWE1 fed (76.67 g/d) and CEP fed (47.50 g/d) piglets. Piglets in SDWE groups grew faster than the CEP piglets with the gain/feed ratio (G:F) being significantly ($P = 0.015$) higher in SDWE2-fed pigs than in CEP group. However, no significant difference was observed in ADFI among treatments. Seven days post ETEC $K88^+$ oral inoculation (wk 2), ADG ($P = 0.920$), ADFI ($P = 0.596$) and G:F ($P = 0.790$) were similar among dietary treatments.

The weaning transition is one of the most stressful events a young pig encounters in swine production (Xu et al., 2014) with consequent reductions in growth performance (Boudry et al., 2002, 2004; Heo et al., 2013), compromised intestinal health (Moeser et al., 2007), diarrhea (Heo et al., 2013) and high mortality rate (Madec et al., 2000) resulting in significant economic losses, and ETEC expressing F4 fimbrial antigens is an important etiology of PWD in pigs (Fairbrother et al., 2005). Studies by Zyme Fast System have confirmed that the F4 gene was present in *E. coli* high expression system used to express the antigen, that the system expressed a protein having the same molecular weight as the F4 antigen, and that their antigen and the wild type antigen had identical reactivities in a competitive ELISA using an anti-F4 antibody that was supplied by Dr. Fairbrother, University of Montreal, Quebec, Canada. This antibody was produced against purified wild type F4 fimbriae. Zyme Fast System demonstrated that its new antibodies produced against

the recombinant F4 antigen had an identical ability to bind to both the wild type and the recombinant antigens, and that of the whole ETEC-F4 organism.

Therefore, we hypothesized that supplementing pig starter diet with spray-dried whole egg powder containing anti-F4 antibodies (SDWE) against recombinant F4 antigens would improve growth performance and reduce ETEC K88-induced diarrhea in early-weaned piglets. The growth performance results observed in the current study are similar to observations previously made in 21 d old weaned piglets fed diets containing egg yolk antibodies (Marquardt et al., 1999; Owusu-Asiodu et al., 2002). In wk 1, feeding egg antibodies numerically (20%) increased ADFI in SDWE2 group compared to piglets fed control diet.

Table 2. Effects of spray-dried whole egg powder containing anti-F4 antibodies (SDWE) on piglet growth performance

	Treatment[1]				
Parameter	CEP	SDWE1	SDWE2	SEM	*P*-value
Initial BW, g	7300	7250	7230	163.7	0.961
Final BW, g	8700	8820	9180	259.0	0.405
ADG, g					
1 to 7 d	47.50	76.67	110.74	13.89	0.011
8 to 14 d	203.33	199.63	214.07	25.90	0.920
ADFI, g					
1 to 7 d	137.23	143.83	164.61	10.69	0.170
8 to 14 d	261.07	255.63	285.32	21.72	0.596
G : F					
1 to 7 d	0.34	0.49	0.67	0.08	0.015
8 to 14 d	0.78	0.75	0.72	0.05	0.790

SEM: Standard error of the mean

[1]CEP = basal diet plus 4 kg control egg powder/ton feed (0%); SDWE1 = basal diet plus 1 kg whole egg powder containing anti-F4 antibodies and 3 kg control egg powder/ton feed (0.1%); SDWE2 = basal diet plus 4 kg whole egg powder containing anti-F4 antibodies/ton feed (0.4%).

Seven days post ETEC K88^{+} oral inoculation (wk 2), ADG and G:F were similar among dietary treatments ($P > 0.05$). The ADFI was not significantly affected ($P > 0.05$) during d 1 to d 7 and d 8 to d 14 by dietary treatment but piglets fed SDWE containing anti-F4 antibodies diet had better ADFI (numerically) compared to CEP piglets. This could therefore suggest that egg antibodies had appetite enhancing effect that may be attributable to the presence of the specific antibodies and presumably, enabled antibody-fed piglets to consume more feed hence, better growth rate. In pigs, a number of studies (e.g. Yokoyama et al., 1992; Yokoyama et al., 1997; Imberechts et al., 1997; Marquardt et al., 1999; Liou et al., 2011) have demonstrated positive effect of egg anti-*E. coli* antibodies on the reduction of diarrhea and mortality whereas a few studies (e.g. Marquardt et al., 1999; Owusu-Asiodu et al., 2002; Heo et al., 2015) reported effects on growth performance response criteria with inconsistent results. In the current study, although the growth-promoting effect of SDWE was evident during the pre-challenge period, such benefit was not observed after *E. coli* challenge contrary to observations reported by previous studies (e.g. Marquardt et al., 1999; Owusu-Asiodu et al., 2002) but partly in agreement to recent observations made by Heo et al. (2015). In their study, Heo and colleagues (2015) reported that egg antibodies did not significantly affect growth response parameters of 21-d-old piglets in the first phase (14-d period and unchallenged) of the investigation but increased the ADFI and tended to increase the ADG in the second phase (11-d period and unchallenged) when piglets were fed a common commercial diet. This may then suggest a carry-over effect from the phase I of supplementing piglet diet with egg antibodies. Dosages, mode of administration (in-feed versus liquid), breed, age and study conditions (e.g. challenge vs. unchallenged) may partly explain differences between our results and previous observations. Hence, this warrants more investigations into the mechanism underlying growth performance-improving property of chicken egg antibodies.

The PUN content was not affected by any of the dietary treatments during the pre-challenge (P = 0.471) and 24 h (P = 0.742), 48 h (0.391) and d 7 (P = 0.145) post-*E. coli* oral challenge (Table 3). Also the serum concentrations of IL-6 (pre-challenge; P = 0.536, 24 h; P = 0.717, 48 h; P = 0.630) and TNF-α (pre-challenge; P = 0.746, 24 h; P = 0.458, 48 h; P = 0.218) were not significantly different among treatments (Table 3). At d 2 (48 h) post challenge, PUN levels increased ($P > 0.05$) across the dietary treatments compared to d 1 (24 h) after inoculation and may indicate evidence of body protein breakdown (catabolism) for energy use and an inefficient utilization of dietary protein for protein synthesis as a result of deleterious effects of pathogenic microorganisms (Coma et al., 1995). This may as well support no significant differences observed in growth performance parameters among the three treatments after ETEC-K88 challenge. The IL-6 seems to be a potential marker for ongoing bacterial infections in pigs (Fossum et al., 1998) because of its upregulation particularly during ETEC infection (Zhang et al., 2010). In our study, serum IL-6 concentration at 24 h post-challenge was lower (numerically) in piglets fed diet containing SDWE2 relative to the CEP group. This may partly explain reduced incidence and severity ($P > 0.05$) of diarrhea observed in SDWE2-fed piglets compared with the control group. On the other hand, serum TNF-α level was numerically upregulated in SDWE2 group and this may be beneficial because TNF-α has been shown to upregulate the expression of immunoglobulin secretory component responsible for the transcytosis of newly synthesized IgA that seems to play important roles in regulating eosinophil functions and enhancing local immune responses (Liu et al., 2007).

Table 3. Effects of spray-dried whole egg powder containing anti-F4 antibodies (SDWE) on PUN content and serum concentrations of IL-6 and TNF-α in ETEC-K88 challenged piglets

	Treatment[1]				
Item	CEP	SDWE1	SDWE2	SEM	*P*-value
IL-6, pg/μL					
0 h	147.82	90.85	94.69	28.72	0.310
24 h	163.57	134.40	106.63	38.49	0.586
48 h	134.15	172.79	135.06	41.90	0.760
TNF-α, pg/μL					
0 h	285.68	229.71	233.27	57.56	0.746
24 h	168.64	163.41	232.57	42.86	0.458
48 h	114.38	204.03	168.66	59.59	0.571
PUN, mmol/L					
0 h	5.06	5.24	4.51	0.43	0.471
24 h	3.48	3.38	3.94	0.55	0.742
48 h	4.22	3.83	4.98	0.59	0.391
d 7	3.86	2.84	4.26	0.50	0.145

SEM: Standard error of the mean

[1]CEP = basal diet plus 4 kg control egg powder/ton feed (0%); SDWE1 = basal diet plus 1 kg whole egg powder containing anti-F4 antibodies and 3 kg control egg powder/ton feed (0.1%); SDWE2 = basal diet plus 4 kg whole egg powder containing anti-F4 antibodies/ton feed (0.4%).

As shown in Table 4, 24 h after ETEC challenge, mild to severe diarrhea was observed in 3 piglets from CEP group while 2 each from SDWE1 and SDWE2-fed piglets had mild diarrhea. By d 2 (48 h) post-challenge, SDWE-fed piglets recovered from diarrhea compared to control diet-fed group with 1 severe diarrheic piglet till d 4. The results show that 0.1% and 0.4% inclusion levels of SDWE had a similar effect ($P > 0.05$) on reducing ETEC K88-induced diarrhea.

Twenty-four hours after oral ETEC $K88^+$ challenge, 33% (3/9) developed severe diarrhea in CEP fed piglets as compared to 22% (2/9) in SDWE fed piglets with mild diarrhea. After 48 h (post challenge), all diarrheic piglets (100%) in egg antibody-supplemented groups recovered from the mild diarrhea while 11% (1/9) in CEP group did not recover from severe diarrhea after d 4. Hence, adding egg powder containing anti-F4 antibodies was able to reduce incidences and severity of diarrhea and increase recovery rate in piglets ($P > 0.05$) experimentally infected with ETEC $K88^+$. Although the differences in incidences and severity of diarrhea were not statistically significant ($P > 0.05$) compared to placebo (CEP-fed), supplementing piglet diet with egg anti-F4 antibodies resulted in a mild diarrhea, rapid recovery and 100% survival rate (Marquardt et al., 1999; Owusu-Asiodu et al., 2002). The diarrhea-reducing effects of chicken egg anti-ETEC antibodies are inconsistent and have been demonstrated and reported to be dose-dependent by a number of research studies (e.g. Yokoyama et a., 1992; Erhard et al., 1996; Yokoyama et a., 1997; Imberechts et al., 1997; Marquardt et al., 1999). For example, Yokoyama et al. (1997) reported protective effects of egg yolk antibodies against *E. coli* induced diarrhea when each piglet was fed 5.5 g per day but no effect with 3.5 g per pig per day whereas Marquardt et al. (1999) demonstrated positive effect with 1.5 g egg yolk powder antibodies per pig daily for 2 days post experimental infection. Also Imberechts et al. (1997) used 30 g of egg yolk powder per pig daily to prevent (100%) experimentally induced PWD.

Table 4. Incidences of diarrhea in piglets fed control egg powder and egg powder containing anti-F4 antibodies (SDWE)

	No. of piglets with diarrhea/total (FC score)[a]			
	Treatment[1]			
Post-challenge	CEP	SDWE1	SDWE2	*P*-value
d 1	3/9 (2.3)	2/9 (2.0)	2/9 (2.5)	0.867
d 2	1/9 (3.0)	0/9 (0.0)	1/9 (2.0)	0.607
d 3	1/9 (3.0)	0/9 (0.0)	0/9 (0.0)	0.368
d 4	1/9 (3.0)	0/9 (0.0)	0/9 (0.0)	0.368

[1]CEP = basal diet plus 4 kg control egg powder/ton feed (0%); SDWE1 = basal diet plus 1 kg whole egg powder containing anti-F4 antibodies and 3 kg control egg powder/ton feed (0.1%); SDWE2 = basal diet plus 4 kg whole egg powder containing anti-F4 antibodies/ton feed (0.4%).

[a]FC score is the mean fecal consistency score: 0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea. ≤ 1 fecal consistency score means no diarrhea.

Table 5. Viable counts of *E. coli* $K88^+$ in fecal and ileal digesta samples and ileal histomorpholgy of piglets fed control egg powder and egg powder containing anti-F4 antibodies (SDWE)

	Treatment[1]				
Item	CEP	SDWE1	SDWE2	SEM	*P*-value
d 7, feces (log_{10} cfu/mL)	7.5	7.3	5.9	1.07	0.320
d 7, digesta (log_{10} cfu/mL)	7.8	6.3	8.2	0.51	0.056
VH (µm)	325.1	317.0	309.8	16.80	0.708
CD (µm)	263.4	264.6	276.0	14.40	0.816
VH:CD	1.25	1.21	1.16	0.09	0.749

SM: Standard error of the mean

[1]CEP = basal diet plus control egg powder/ton feed (0%); SDWE1 = basal diet plus 1 kg spray-dried whole egg powder containing F4 antibodies and 3 kg control egg powder/ton feed (0.1%); SDWE2 = basal diet plus 4 kg spray-dried whole egg powder containing F4 antibodies/ton feed (0.4%); VH = villus height; CD = crypt depth; VH:CD = villus height-crypt depth ratio.

In contrast, Chernysheva et al. (2004) reported no significant diarrhea-reducing effects when each piglet consumed daily approximately 13 g egg yolk powder containing anti-F4 antibodies. They concluded that even at high inclusion rates, egg yolk antibodies may not be efficacious in 3 to 4 weeks old pigs. Gastric pH and digestive enzyme activities are among reasons suggested by these authors to be likely responsible for no significant effects as observed.

In the present study, each piglet consumed less than 450 g (i. e. post-challenge) of feed per day, meaning less than 1.8 g SDWE daily for the high-dose (0.4%, SDWE2) category and challenged with 2 x 10^9 cfu/ml ETEC $K88^+$ inoculum less than those used in above cited studies of 10^{10} to 10^{12} cfu/ml ETEC inoculum. The oral challenge model used in the present study was insufficiently sensitive to induce responses similar to clinical cases of PWD in the challenged piglets. This may partly explain why our results differed with no significant differences in both growth-enhancing and diarrhea-reducing effects of SDWE antibodies after ETEC $K88^+$ inoculation. Also, for the assessment and evaluation of protective effects of orally administered egg antibodies, a certain nutritive effect of egg powder may be considered (Gurtler et al., 2004). For example, O'Farrelly et al. (1992) demonstrated protective effect in rabbits against artificial infection by *E. coli* after administering egg yolk powder obtained from non-immunized laying hens. Therefore, non-significant effects recorded in our study may be due to some nutritive effects of egg powder from non-immunized laying hens (control egg powder), although small amounts were consumed. This would require further studies as to whether egg powder from non-immunized or egg white from immunized laying hens has some protective effect against the diarrheal disease caused by pathogenic *E. coli*.

The fecal shedding of ETEC $K88^+$ (Table 5) d 7 post-challenge was numerically reduced ($P > 0.05$) in SDWE2 fed piglets compared to CEP fed piglets (5.9 vs. 7.5, respectively) while

ETEC K88^{+} recovery from ileal digesta was significantly lower ($P < 0.05$) in piglets fed diet containing SDWE1. This may explain mild diarrhea and quick recovery observed in piglets fed diet containing SDWE similar to observations made by Yokoyama et al. (1992) and Marquardt et al. (1999). Results of ileal histomorphology (Table 5) were similar among the three treatment diets. Comparing data on intestinal morphology from different experiments is difficult because of differences in the diets, breed, age, experimental conditions and as well as no known standards for the measurements of VH and CD (Heo et al., 2013). Nevertheless, within experiments of similar conditions, data may be compared and some deductions made as previous studies (e.g. Hornich et al., 1973; Cera et al., 1988; Pluske et al., 1997; McCracken et al., 1999) have associated reduced VH and increased CD to reduced feed intake, post-weaning growth lag and diarrhea in early weaned pigs. But in the present study, the results of ileal morphology were similar among the three treatments correlating well with observed growth performance.

Under the conditions of the present study, it can be concluded that supplementation of piglet diets with 0.4% SDWE anti-F4 antibodies supported greater performance during the pre-challenge period but this benefit was not evident at either 0.1% or 0.4% inclusion level of SDWE during the post-challenge period in 21-d-old piglets.

CHAPTER FOUR

MANUSCRIPT II

Combined effects of chitosan and probiotic supplementation on performance and diarrhea incidences in enterotoxigenic *Escherichia coli* K88^{+} challenged piglets

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ABSTRACT

The aim of this study was to investigate the combined effects of chitosan oligosaccharide (COS) and a microencapsulated *Enterococcus fecalis* probiotic (PRO) on growth performance and diarrhea incidences in enterotoxigenic *Escherichia coli* K88 (ETEC) challenged piglets in a 14-d study. Thirty piglets [(Yorkshire x Landrace) x Duroc], 7.19 ± 0.52 kg initial BW weaned at 21 ± 1 d were allotted to 5 treatment groups (n = 6) consisting of a corn-soybean meal diet with no additive (negative control, NC), the NC + 0.25% chlortetracycline (positive control, PC), NC + 400 mg/kg COS (COS), NC + 100 mg/kg PRO (PRO) and NC + a combination of COS and PRO (CPRO). The basal diet was formulated to meet the NRC (2012) nutrient specifications for 5 to 10 kg BW pigs. Pigs were individually housed in cages, acclimated to treatments for a 7-d period and had *ad libitum* access to feed and water throughout the study. On d 8, pigs were weighed, blood samples were collected and then orally challenged with 6 mL (1 x 10^{11} cfu/mL) of freshly grown ETEC inoculum. Post-challenge, blood was sampled at 24 h and 48 h to determine plasma urea nitrogen (PUN) and diarrhea incidences and fecal consistency scores were recorded from d 9 to d 12. On d 14, all pigs were weighed and then euthanized to obtain intestinal tissue samples for histomorphometric measurements. Growth performance was similar among treatments during the pre- and post- challenge periods. There were no significant differences in PUN content, incidences of diarrhea and fecal consistency scores among treatments. The intestinal histomorphology results did not differ significantly among treatments except for PC with increased (P = 0.0003) villus-crypt ratio compared with the NC. Therefore, under the conditions of the present study, it can be concluded that supplementation of piglet diets with 400 mg/kg chitosan oligosaccharide, 100 mg/kg microencapsulated *Enterococcus fecalis* probiotic or their combination neither significantly improved piglet growth performance both during the pre- and post-ETEC $K88^+$ oral inoculation

nor reduced incidences and severity of diarrhea after challenge in early-weaned piglets compared to the control group.

Keywords: ETEC K88^{+}, chitosan oligosaccharide, *Enterococcus fecalis* probiotic, piglets

INTRODUCTION

Infection with enterotoxigenic *Escherichia coli* expressing the F4 (K88^{+}) fimbriae (ETEC F4) is one of the most important causes of PWD in pigs with significant economic losses (Fairbrother et al., 2005; Daudelin et al., 2011) resulting from reductions in performance (Boudry et al., 2002, 2004), compromised intestinal health (Moeser et al., 2007), increased susceptibility to diseases and high mortality rate (Madec et al., 2000). It has been shown that colonization of the small intestine of the pig by ETEC adhering to the epithelium accounts for most gastrointestinal disorders in both neonatal and post-weaning piglets (Yokoyama et al., 1992; Marquardt et al., 1999). Overtime, this challenge has been managed by in-feed sub-therapeutic administration of AGPs. However, there are concerns about in-feed antibiotics due to antimicrobial drug residues in food animal products and increased antibiotic resistant bacteria (Diraviyam et al., 2014), leading to increased public pressure to suspend the use of in-feed antibiotics in livestock diets (Hulst et al., 2013), hence the need for identifying effective and viable alternative therapies (Owusu-Asiedu et al., 2003a; Kiarie et al., 2009, 2011). And any replacement for AGPs would have to provide an improvement in performance and feed efficiency that is economically viable and a combination of candidate alternatives must be identified (Dibner and Richards, 2005). One of these promising alternative therapies is a combination of chitosan oligosaccharide (COS) and *Enterococcus fecalis* probiotic (PRO) [CPRO] because of a possible synergy of actions coupled with enhanced effects between these two additives in protecting early-weaned piglets against deleterious effects of ETEC-K88 infection.

Chitosan is a linear polysaccharide composed of randomly distributed beta (1-4) –linked D-glucosamine and N-acetyl-D-glucosamine that has inhibitory effects on *E. coli* in pigs (Sun Haixiang et al., 2005), reducing incidence of diarrhea and dependence on antimicrobials in weaned piglets. It has also been shown to improve growth performance and nutrient digestibility in weaned piglets (Xu et al., 2014). Being a polycationic molecule (Rabea et al., 2003), chitosan binds to the predominantly anionic cell surface of Gram-negative (G-ve) bacteria such as *E. coli* resulting in changes in outer membrane permeability and subsequent leakage of cell constituents such as enzymes and glucose thus, preventing its growth and spread and rendering *E. coli* more sensitive to the inhibitory action of bile and organic acids. Binding of polycationic molecules to bacterial cell wall has been shown to disrupt the integrity of the outer membrane resulting in loss of the barrier function (Helander et al., 2001), destabilization of cell membrane, leakage of intracellular substances, and ultimately, the death of cells (Kong et al., 2010).

On the other hand, probiotics are live microbial agents that have beneficial effects on the intestinal microbial balance of the host and are an effective factor to favorable health and functionality of the GIT. Various strains of bacteria have been used as probiotics and the most commonly used species include *Bacillus,* yeast and lactic acid-producing bacteria such as *Lactobacillus*, *Streptococcus*, *Bifidobacterium* and *Enterococcus* (Stein and Kil, 2006; Bednorz et al., 2013). The volatile fatty acids e.g. lactic acid produced by these probiotic bacteria possess potent bactericidal activity against members of *Enterobacteriacae* (Brocklehurst and Lund, 1990). Also they act competitively by exclusion in which attachment of probiotic microorganisms on the intestinal epithelial surfaces prevents pathogens such as *E. coli* from attaching (Stein and Kil, 2006).

Therefore, the objective of the present study was to determine growth performance and incidences of diarrhea in ETEC K88^{+}-challenged piglets when fed diets containing a combination of COS and PRO (CPRO).

MATERIALS AND METHODS

Animals, treatments and oral challenge

The experimental protocol was approved by the Animal Care Committee of the University of Manitoba. Pigs were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

Thirty piglets [(Yorkshire x Landrace) x Duroc, initial BW of 7.19 ± 0.52 kg] weaned at 21 ± 1 days of age from the University of Manitoba's Glenlea swine research unit were used in this study. Pigs were individually housed in cages (dimensions: 2.5 Ft. x 2 Ft. x 15 in.) within a room in a 14-d trial at the T. K. Cheung Centre for Animal Science Research, University of Manitoba, Winnipeg, Canada. Room temperature was maintained at 30 ± 1^{o}C throughout the experimental period. Piglets were allotted to 5 treatment groups (n = 6) consisting of a corn-soybean meal diet with no additive (negative control, NC), the NC + 0.25% chlortetracycline (positive control, PC; Alpharma Canada Corporation, Mississauga, Ontario, Canada), NC + 400 mg/kg COS (COS, degree of deacetylation > 90%; Dalian GlycoBio Company Ltd, Dalian, Liaoning, China), NC + 100 mg/kg PRO (PRO; SKF Biotechnology Company Ltd, Beijing, China) and NC + a combination of COS and PRO (CPRO). The basal diet (Table 5) was formulated to meet the NRC (2012) nutrient specifications for 5 to 10 kg BW pigs. Feed and water were provided *ad libitum* and after a 7-d period of adaption, pigs were weighed, blood samples collected (venipuncture via the jugular vein) to determine plasma urea nitrogen (PUN) content and then orally challenged with 6 mL (1 x 10^{11} cfu/mL) of freshly grown ETEC inoculum. Body weights

(BW) and feed intakes were determined weekly and average daily BW gain (ADG), average daily feed intake (ADFI) and the ratio of BW gain to feed intake (G: F, i.e., feed conversion efficiency, FCE) were calculated. Pigs were monitored for another 7 d post-challenge for incidences of diarrhea, feed intake, BW gain and general health conditions. Incidences and severity of diarrhea were assessed on a cage basis (individual animal basis) by two trained independent personnel (without prior knowledge of dietary treatment allotment) using a fecal consistency scoring system [0 = normal feces; 1 = soft feces; 2 = mild diarrhea; 3 = severe diarrhea (Marquardt et al., 1999)]. The fecal consistency for each piglet was determined by averaging the assigned scores by two independent personnel and a score of ≤ 1 was considered to indicate no diarrhea.

ETEC K88$^+$ and culture condition

The ETEC-F4 strain was originally obtained from Veterinary Diagnostic Services of Manitoba, Winnipeg, Manitoba, Canada. From the frozen stock, *E. coli* K88$^+$ was streaked on brain heart infusion (BHI) agar and grown anaerobically at 37^{o}C overnight. Then a single colony was inoculated on two BHI plates (i.e. duplicate) and incubated anaerobically at 37^{o}C overnight. Two tubes of 5 ml BHI broth (BD & Co., Franklin Lakes, New Jersey, USA) plus 2% casamino acids (Fisher Scientific, Waltham, MA, USA) were inoculated from a single colony and grown overnight at 37^{o}C with shaking (200 rpm). The *E. coli* K88$^+$ identity was verified using an *E. coli* K88 fimbrex latex agglutination kit. Two flasks of 500 ml BHI broth plus 2% casamino acids were inoculated with 2 ml *E. coli* K88$^+$ from the 5 ml culture tube and then incubated anaerobically at 37^{o}C overnight with shaking (200 rpm). The two 500 ml flasks were combined and thoroughly mixed. With serial dilution of the culture 10-fold in PBS, 10^6 to 10^9 dilutions were plated on BHI plates to check that the culture was > 1 x $10^{\wedge 9}$. Incubation was done anaerobically at 37^{o}C

overnight. The colonies on the dilution plates were counted the following day to determine concentration and 6 ml of 1 x 10^{11} cfu/ml per piglet was used for inoculation.

Table 6. Composition and calculated nutrient levels of basal diet (d 21 to d 35, as-fed basis)

Item	%
Ingredients	
Corn	14.35
Wheat HRW	30.00
Soybean meal	28.00
Dried whey	19.00
Vegetable oil	5.00
Limestone	0.77
Calcium monophosphate	0.76
Iodized salt	0.42
Vitamin-mineral premix*	1.00
Lys-HCl	0.33
DL- methionine	0.20
Threonine	0.14
Tryptophan	0.03
Total	100.00
Calculated nutrient levels	
ME, kcal/kg	3430
CP	20.84
Lys	1.49
Met	0.50
Met + Cys	0.87
Thr	0.95
Trp	0.30
Ca	0.78
Av.P	0.40

*Vitamin-premix provided per kg of diet: vitamin A 8,250 IU, vitamin D3 835 IU, vitamin E 40 IU, vitamin K3 4 mg, vitamin B12 0.025 mg, vitamin B1 2 mg, vitamin B2 12 mg, nicotinic acid 22.5 mg, folic acid 2 mg, pyridoxine 4.5 mg, biotin 0.2 mg, pantothenate 15 mg, choline 500 mg, Mn 50 mg, Fe 100 mg, I 0.4 mg, Cu 25 mg, Zn 150 mg, Se 0.3 mg

Blood sample collections

Heparinized blood samples to obtain plasma for plasma urea nitrogen (PUN) concentration analysis were collected from the jugular vein of each piglet on d 8 (before *E. coli* oral inoculation), d 1, and 2 (post *E. coli* challenge). Samples were immediately centrifuged at 2,000 x g for 10 min at 5^{o}C to harvest plasma and stored at -20^{o}C until required for PUN analysis.

Intestinal tissue collection

Ileal sections were collected from all the piglet on d 7 post ETEC challenge after being anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and euthanized by an intracardiac injection of sodium pentobarbital (50 mg/kg of body weight; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada). Fifteen centimeters from the ileocecal junction, a 2 cm ileal section was collected from each piglet and stored in 10% formalin to fix the villus and crypt for subsequent histomorphometric measurement. Cross-sections from formalin-fixed samples were processed for histological examination using the standard Hematoxylin and Eosin (H&E) method. Measurement of villus height (VH) and crypt depth (CD) was made on at least ten well-oriented villi per specimen using a Zeiss photomicroscope equipped with a Sony 3 chip CCD color camera (Carl Zeiss, Oberkochen, Germany). Captured images were analyzed using NIH ImageJ software (NIH Image, Bethesda, Maryland, USA) with the VH being measured from the tip to the villus-crypt junction and the CD from this junction to the base and villus height to crypt depth ratio (VH:CD) determined.

Statistical analysis

Data were subjected to statistical analysis of the Mixed Procedure of SAS, 9.4 version (SAS Institute Inc., Cary, NC, USA). Cage was the random effect and diets (main effects of NC,

PC, COS, PRO and CPRO) were the fixed effects. Means for significant treatment differences were compared by the least significant difference (LSD) test. Probability values of $P \leq 0.05$ were considered statistically significant. Chi-square test was performed on diarrhea incidences to determine if differences among the treatment groups were significant or not with significant levels being considered at $P \leq 0.05$.

RESULTS AND DISCUSSION

The effects of dietary treatments on ADG, ADFI and G: F ratio are presented in Table 7. Final BW was similar among treatments and dietary supplementation with COS, PRO or their combination (CPRO) had similar effects on growth performance both during the pre- and post-ETEC challenge periods. Final BW ranged from 9.24 kg (COS-fed piglets) to 10.38 kg (PC group). ADG, ADFI and G:F were not affected ($P > 0.05$) by the treatments pre-ETEC $K88^+$ oral challenge (d 1 to d 7). Day 8 to d 14, CPRO-fed group had the highest G:F and was significantly different ($P < 0.05$) from the NC group. ADFI was not affected ($P > 0.05$) by the treatments. The effects of chitosan on growth performance of broilers, pigs or other livestock species are not consistent (Xu et al., 2014). For example, Suk (2004) and Khambualai et al. (2008, 2009) attributed superior performance and feed conversion ratio obtained in broilers to dietary chitosan supplementation when compared with the control group whereas Razdan et al. (1997) observed significantly reduced body weights and feed intake of broiler chickens fed 30 g/kg chitosan compared with those fed control diets. In pigs, Tang et al. (2005), Walsh et al. (2013), Xu et al. (2014) and Xiao et al. (2014) reported growth-promoting effects of chitosan and these were attributed to increased feed intake (Yuan and Chen, 2012), increased apparent digestibility of nutrients (Lim et al., 2006; Liu et al., 2008; Chen et al., 2009), reduced incidence of diarrhea and improved small intestinal

morphology (Liu et al., 2008). However, non-positive effects on nutrient digestibility had been reported by some authors (e.g. Razdan and Patterson, 1994, 1996; O'Shea et al., 2011).

The positive gut health effect of chitosan may be linked to its microbial activity against pathogenic microorganisms particularly the Gram-negative (G -ve) bacteria such as *E. coli* via electrostatic interaction with bacterial cell wall and membrane (Rabea et al., 2003; Kong et al., 2010). This interaction between chitosan and bacterial cells causes loss of cell wall protection and exposure of cell membrane leading to drastic increased membrane permeability (Kong et al., 2008a). The binding also promptly neutralizes and even reverses the surface charge of the bacteria (Chen and Cooper, 2002), destabilization of cell membrane and leakage of intracellular substances, finally, the cell death (Kong et al., 2010).

However, in the present study, COS alone did not improve growth performance pre- and post-ETEC $K88^{+}$ oral challenge in weaned piglets and this may be attributed to decreased feed intake as observed in our study because feed consumption is one of the major factors limiting growth in young pigs as previously suggested by Yuan and Chen (2012) that growth performance was improved with dietary chitosan supplementation as a result of increased feed intake. The observed similar effects on growth performance in CPRO-fed piglets compared to COS or PRO group can partly be explained by some antibacterial activity of chitosan on Gram-positive (G +ve) bacteria such as *Enterococcus fecalis* (used as probiotic in the present study). Nevertheless, the inhibitory effect has been reported to be more pronounced on G -ve bacteria because of the possession of higher negative charges (polyanions) on cell surface and adsorption of more chitosan on to the G -ve bacterial cell wall (Chung et al., 2004).

The cell wall of G +ve bacteria comprises peptidoglycan and teichoic acid. Teichoic acid is an essential polyanionic polymer of the cell wall traversing the wall to contact with the

peptidoglycan layer (Kong et al., 2010). Linkage between chitosan and cell surface via electrostatic interaction with the teichoic acid allows chitosan to disturb membrane functions (Raafat et al., 2008) and can subsequently lead to cell death (Kong et al., 2010).

Table 7. Growth performance of piglets fed COS, PRO and a combination of COS and PRO (CPRO)

	Treatment[1]						
Item	NC	PC	COS	PRO	CPRO	SEM	*P*-value
IBW, g	7210	7120	7210	7230	7200	227.8	0.999
FBW, g	9940	10380	9480	10300	9240	483.5	0.521
ADG, g							
1 to 7 d	156	176	105	162	97	40.06	0.547
8 to 14 d	299	367	291	350	353	40.77	0.577
ADFI, g							
1 to 7 d	214	215	162	208	139	33.82	0.558
8 to 14 d	431	447	348	449	382	41.52	0.460
G:F							
1 to 7 d	0.62	0.78	0.59	0.86	0.61	0.12	0.375
8 to 14 d	0.69	0.83	0.81	0.79	0.94	0.07	0.273

SEM: Standard error of the mean

[1]NC: Basal diet (wheat-corn based) as negative control; PC: NC plus 2.5 g chlortetracycline/kg feed; COS: NC plus 400 mg chitosan oligosaccharide (COS)/kg feed; PRO: NC plus 100 mg *Enterococcus fecalis* probiotic (1.0×10^{10} cfu/g microcapsules)/kg feed; CPRO: NC plus 400 mg COS/kg feed and 100 mg *Enterococcus fecalis* probiotic (microcapsules)/kg feed.

IBW = initial body weight; FBW = final body weight.

Table 8. Diarrhea incidence in piglets fed COS, PRO and a combination of COS and PRO (CPRO)

	No. of piglets with diarrhea/total (FC score)*					
	Treatment[1]					
Post-challenge	NC	PC	COS	PRO	CPRO	*P*-value
12 h	2/6 (2.0)	0/6 (0.0)	1/6 (2.0)	2/6 (2.0)	1/6 (2.0)	0.549
d 1	4/6 (2.5)	1/6 (2.0)	1/6 (2.0)	2/6 (2.0)	3/6 (2.0)	0.314
d 2	2/6 (2.0)	0/6 (0.0)	1/6 (2.0)	2/6 (2.0)	3/6 (2.0)	0.356
d 3	3/6 (2.0)	0/6 (0.0)	0/6 (0.0)	1/6 (2.0)	1/6 (2.0)	0.144

[1]NC: Basal diet (wheat-corn based) as negative control; PC**:** NC plus 2.5 g chlortetracycline/kg feed; COS: NC plus 400 mg chitosan oligosaccharide (COS)/kg feed; PRO: NC plus 100 mg *Enterococcus fecalis* probiotic (1.0×10^{10} cfu/g microcapsules)/kg feed; CPRO: NC plus 400 mg COS/kg feed and 100 mg *Enterococcus fecalis* probiotic (microcapsules)/kg feed.

*The FC score is the mean fecal consistency score: 0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea. ≤ 1 fecal consistency score means no diarrhea.

Table 9. The PUN content and ileal histomorphology of piglets fed COS, PRO and a combination of COS and PRO (CPRO)

	Treatment[1]						
Item	NC	PC	COS	PRO	CPRO	SEM	*P*-value
PUN, mmol/L							
0 h	1.92	1.58	1.10	1.77	1.30	0.33	0526
24 h	2.97	2.23	3.22	2.38	3.23	0.46	0.530
48 h	2.87	2.02	2.62	2.37	2.55	0.46	0.821
VH, µm	283.8	359.7	320.3	310.3	301.0	17.72	0.0994
CD, µm	262.5	206.7	260.0	254.0	247.5	17.22	0.2526
VH:CD	1.07	1.76	1.28	1.24	1.25	0.09	0.0003

SEM: Standard error of the mean

[1]NC: Basal diet (wheat-corn based) as negative control; PC**:** NC plus 2.5 g chlortetracycline/kg feed; COS: NC plus 400 mg chitosan oligosaccharide (COS)/kg feed; PRO: NC plus 100 mg *Enterococcus fecalis* probiotic (1.0 x 10^{10} cfu/g microcapsules)/kg feed; CPRO: NC plus 400 mg COS/kg feed and 100 mg *Enterococcus fecalis* probiotic (microcapsules)/kg feed.

Although supplementing piglet diet with PRO alone did not significantly improve piglet growth performance, numerically, the probiotic piglets had better growth performance than COS fed piglets (pre-challenge: ADG = 162 g vs. 105 g; ADFI = 208 vs. 162; G:F = 0.86 vs. 0.59; post-challenge: ADG = 350 vs. 291; ADFI = 449 vs. 348; G:F = 0.79 vs. 0.81, respectively). This may suggest that there was no synergistic action between COS and PRO, hence non-significant effect on growth performance response criteria as observed.

Table 9 shows the PUN results demonstrating that no significant effects were observed before challenge and 24 h and 48 h after ETEC challenge although the PUN levels increased across treatment groups from the baseline (0 h) level at 24 h post-inoculation. However, at 48 h after challenge, there was gradual reduction of PUN content. The transient PUN elevations may be attributed to negative effects of ETEC on energy metabolism as a result of inefficient utilization of diet protein and body protein breakdown for synthesis of acute phase proteins in the hepatocytes (Coma et al., 1995).

Effects of dietary treatment on incidences and severity of diarrhea are shown in Table 8 indicating no significant differences among dietary treatments. Twelve hours post ETEC $K88^{+}$ oral inoculation, 2 (33%) piglets each from NC and PRO fed groups developed mild diarrhea whereas 1 (17%) each from COS and CPRO groups had diarrhea. The PC-fed piglets did not develop diarrhea. On d 1(24 h) post-challenge, 4 (67%) piglets were observed to have severe diarrhea in NC whereas 3 (50%) in CPRO groups, 2 (33%) in PRO and 1 (17%) each in PC and COS-fed piglets had mild diarrhea. On d 3 after oral *E. coli* inoculation, there were 3 (50%) severe diarrheic piglets in NC-fed piglets and 1 (17%) each in PRO and CPRO-fed piglets with mild diarrhea. From these observations, piglets fed NC and CPRO had the highest incidences and severity of diarrhea compared to PC, COS and PRO fed piglets, hence, supporting the growth performance results and

suggesting no significant synergistic effect of CPRO in reducing ETEC-induced diarrhea in early-weaned piglets.

Table 9 shows the results for small intestinal (ileum) histomorphology in which villus height (VH) tended to differ significantly ($P = 0.099$) among treatment groups but no significant effect ($P = 0.2526$) on crypt depth (CD). The VH:CD was significantly affected ($P = 0.0003$) by treatments. The PC and NC fed piglets had the highest (359.7 µm) and the shortest (283.8 µm) VH, respectively and the PC being significantly different ($P < 0.05$) from NC and CPRO but did not differ significantly from piglets fed diets containing COS and PRO. No significant differences ($P > 0.05$) in VH between the NC and COS or PRO or CPRO fed piglets. The PC fed piglets had shallower CD and differed significantly ($P < 0.05$) NC and COS fed piglets. The VH:CD was significantly affected ($P = 0.0003$) the treatment with PC fed piglets having the highest ratio (1.76) and NC the lowest value (1.07) compared to COS (1.28), PRO (1.24) and CPRO (1.25) groups.

Comparing data on intestinal morphology from different experiments are difficult because of differences in the diets, breed, age, experimental conditions and, as well as, no known standards for the measurements of VH and CD (Heo et al., 2013). Nevertheless, within experiments of similar conditions, data may be compared and some deductions made as previous studies have associated reduced VH and increased CD to reduced feed intake, post-weaning growth lag and diarrhea in early weaned pigs (e.g. Hornich et al., 1973; Cera et al., 1988; Pluske et al., 1997; McCracken et al., 1999). As observed in the present study and in agreement with results of the studies referenced above, NC and CPRO-fed piglets with the shortest VH had higher incidences of diarrhea. Compared with other treatment groups, NC fed piglets had the deepest crypts ($P > 0.05$) probably resulting from crypt hyperplasia for the repopulation of epithelial cells (Zhang and Xu, 2003; Llyod and Gabe, 2008). However, contrary to previous reports (e.g. Liu et al., 2008;

Xiao et al., 2014) that improved intestinal architecture significantly promoted growth performance, no significant growth improvement was observed in our data. These discrepancies may be due to different experimental designs and methodologies, age and breed of animals, genetic factors, types and dosages of additives and anti-nutritive factors in diets.

Under the conditions of the present study, it can be concluded that supplementation of piglet diets with 400 mg/kg chitosan oligosaccharide, 100 mg/kg microencapsulated *Enterococcus fecalis* probiotic or their combination neither significantly improved piglet growth performance both during the pre- and post-ETEC K88 oral inoculation nor reduced incidences and severity of diarrhea after challenge in early-weaned piglets compared to the control group.

CHAPTER FIVE

GENERAL DISCUSSION

The weaning transition period subjects early-weaned piglets to tremendous stress resulting from a combination of factors including sudden interruption of the established social interaction with sow and littermates, adaptation to a new environment, abrupt substitution of sow milk with less digestible diets of plant origin containing complex protein and carbohydrate with anti-nutritional components and loss of maternal immunity (IgA) derived from sow milk (Cranwell, 1995; Lalles et al., 2007). These factors increase the susceptibility of young piglets to enteric infection as caused by ETEC strains that bear the K88, K99 and 987P fimbrial appendages (Jin et al., 1998) but those expressing the K88 fimbrial antigen are the most prevalent forms of *E. coli* infection found globally (Rapacz and Hasler-Rapacz, 1986) estimated to be responsible for 50% of piglet deaths annually (Marquardt et al., 1999). Consequently, intestinal disturbances with diarrhea and depression of growth performance in piglets (Heo et al., 2013). Therefore, reducing diarrhea and improving growth performance in early-weaned piglets are a main challenge for the swine industry (Xu et al., 2014). Antibiotics are routinely included in the diets for weaned pigs to control PWD and optimize growth performance (Verstegen and Williams, 2002). Currently, there are concerns about antimicrobial drug residues in food animal products and the development of a reservoir of antibiotic resistant bacteria that are pathogenic agents in humans (Barton, 2000; Diraviyam et al., 2014), leading to increased public pressure to stop the use of in-feed antibiotics in livestock diets (Hulst et al., 2013). Hence, studies are focusing on identifying effective and viable alternative strategies to antibiotics (Kiarie et al., 2009, 2011) although such replacements must be economically viable for growth performance and identifying a combination of candidate alternatives is of essence (Dibner and Richards, 2005).

In the present research, the ability of spray-dried whole egg powder containing anti-F4 antibodies produced against recombinant F4 antigens (SDWE) to reduce ETEC $K88^+$-induced diarrhea and improve growth performance in 21-d-old piglets was investigated. Also, the combined effects of chitosan oligosaccharide (COS) and *Enterococcus fecalis* probiotic (PRO) [CPRO] on performance and incidences of diarrhea in early-weaned piglets challenged with ETEC-$K88^+$ were investigated. To establish an *E. coli* (ETEC) K88 model of post-weaning diarrhea and to observe responses of piglets to dietary treatments, 21-d-old piglets were orally challenged with freshly grown ETEC-$K88^+$ inocula after a 7-d period of adaptation to treatments. This challenge model has been a robust tool in our lab with which we have been able to rigorously test a large number of additives. However, in the present research and as observed, the challenge model appeared not to work well in creating clinical responses similar to field cases of piglet post-weaning colibacillosis. Nonetheless, results from both the pre- and post-challenge periods of the present research indicated implications of dietary supplementation of SDWE and CPRO in early-weaned piglets.

In manuscript I, the efficacy of SDWE antibodies against recombinant F4 ($K88^+$) antigens in reducing diarrhea and promoting growth performance was tested in 21-d-old piglets challenged with ETEC $K88^+$ in a 14-d study. After the original isolation of F4 fimbrial antigens from wild type strain of ETEC F4 for the immunization of laying hens, it can now be produced recombinantly in a competent *E. coli* high expression system (Zyme Fast System Inc., Winnipeg, MB, Canada). Zyme Fast System Inc. demonstrated that its new antibodies from immunized laying hens produced against the recombinant F4 antigen had an identical ability to bind to both the wild type and the recombinant antigens, and that of the whole ETEC-F4 organism. Therefore, it was hypothesized that supplementing pig starter diet with SDWE would improve growth performance

and reduce incidences and severity of diarrhea in ETEC K88 challenged piglets. Two levels of SDWE (i.e. 0.1% and 0.4%; antibody titre of 8.1 x 10^5) were examined in the present study to compare piglet performance with the control. During the pre-ETEC K88 oral challenge, 0.4% SDWE (SDWE2) had greater ADG and G:F that were significantly different from control egg powder (CEP) fed piglets whereas 0.1% SDWE (SDWE1) fed piglets did not differ significantly from the control in any of the performance response criteria. The ADFI was numerically higher in piglets fed diets containing SDWE relative to control diet-fed piglets. Only a few published studies (e.g. Marquardt et al., 1999; Owusu-Asiodu et al., 2002; Heo et al., 2015) have reported the growth-promoting effect of egg antibodies in pigs whereas effects on diarrhea and mortality have been demonstrated by a number of studies in the literature (Yokoyama et al., 1992; Erhard et al., 1997; Imberechts et al., 1997; Yokoyama et al., 1997; Marquardt et al., 1999). This could therefore suggest that egg antibodies had an appetite enhancing effect that may be attributable to the presence of the specific antibodies and presumably, enabled antibody-fed piglets to consume more feed ($P > 0.05$) hence, better growth rate. On the other hand, after ETEC $K88^+$ challenge, SDWE did not significantly affect piglet growth performance compared to piglets fed diet-containing CEP. The PUN levels increased across the treatments 48 h after challenge compared to 24 h post inoculation partly explaining less use of dietary nutrients for protein synthesis and increased muscle protein breakdown to synthesize acute phase proteins (Wannemacher, 1977). Although incidences of ETEC $K88^+$-induced diarrhea were not significantly different among treatments, piglets fed diets containing SDWE had mild diarrhea and recovered within 48 h compared to those fed CEP in which severe diarrhea was observed and did not recover after d 4 post-challenge. This may demonstrate the protective effect of SDWE against the deleterious consequence of *E. coli* K88 on the intestines by preventing ETEC $K88^+$ attachment and colonization. Also, the fecal

shedding of ETEC K88$^+$ was numerically lower in piglets fed diets supplemented with SDWE compared to control. This may partly explain mild diarrhea and quick recovery observed in piglets fed diets containing SDWE similar to observations made by Yokoyama et al. (1992) and Marquardt et al. (1999).

Serum IL-6 concentration at 24 h post-challenge was lower (numerically) in piglets fed diet containing SDWE2 relative to CEP group probably supporting the reduced incidence and severity ($P > 0.05$) of diarrhea observed in SDWE2-fed piglets compared with the control group. However, serum TNF-α level was numerically upregulated in SDWE2 group and this may be beneficial because TNF-α has been shown to upregulate the expression of immunoglobulin secretory component responsible for the transcytosis of newly synthesized IgA that seems to play important roles in regulating eosinophil functions and enhancing local immune responses (Liu et al., 2007). Intestinal morphometric measurement of VH, CD and VH:CD was not affected by the treatments.

In manuscript II, the objective of the study was to determine combined effects of a combination of COS and PRO (CPRO) on growth performance and incidences of diarrhea in ETEC K88$^+$ challenged piglets. During the pre- and post-challenge periods, growth performance response criteria were not significantly affected by treatments. The G:F was highest in CPRO fed piglets and significantly different from the control but similar among treated groups. The growth-promoting effects of chitosan in broilers, pigs and other livestock species are not consistent (Xu et al., 2014) with some studies reporting significant effects (Suk, 2004; Tang et al., 2005; Khambualai et al., 2008, 2009; Walsh et al., 2013; Xu et al., 2014; Xiao et al., 2014) whereas others observed no effects (Razdan and Patterson, 1994, 1996; Razdan et al., 1997; O'Shea et al., 2011). Among reasons for positive effects on animal performance include increased feed intake (Yuan and Chen,

2012), increased apparent nutrient digestibility (Lim et al., 2006; Chen et al., 2009), reduced incidence of diarrhea and improved small intestine morphology (Liu et al., 2008). The gut health effect of chitosan may be attributed to its microbial activity against pathogenic microorganisms particularly the Gram-negative bacteria such as *E. coli* (Rabea et al., 2003; Kong et al., 2010).

However, in the present study, COS, PRO or their combination did not improve growth performance before and after the challenge and this may be due to decreased feed intake as observed in our results because feed consumption is one of the major factors limiting growth in young pigs (Yuan and Chen, 2012). The non-significant effect of a combination of COS and probiotic (PRO) [CPRO] on growth can partly be explained by some antibacterial activity of chitosan on Gram-positive bacteria such as *Enterococcus fecalis* (used in this study). The cell wall of Gram-positive bacteria comprises peptidoglycan and teichoic acid- an essential polyanions of the cell wall (Kong et al., 2010). Linkage between chitosan and cell surface allows it to disturb membrane function (Raafat et al., 2008) and subsequently, leading to bacterial cell death (Kong et al., 2010). But as observed in the present study, *Enterococcus fecalis* probiotic alone did not significantly improve growth performance pre- and post-ETEC challenge in piglets.

The PUN levels were not significantly affected by dietary treatment, although increases from pre-ETEC challenge were observed across treatments 24 h after inoculation. These transient PUN elevations may be attributed to negative effects of ETEC on energy metabolism resulting from body protein breakdown and inefficient utilization feed nutrients for protein synthesis (Coma et al., 1995). Incidences and severity of diarrhea were similar among treatments, although NC and CPRO-fed piglets had high (numerically) diarrhea incidences and severe diarrhea. This may partly indicate that there was no synergistic effect between COS and PRO probably due to antibacterial action on *Enterococcus fecalis*.

The intestinal histomorphometric measurement results did not differ significantly among treatments except for PC-fed piglets with increased (P = 0.0003) VH:CD compared with the NC-fed piglets. This may further support the non-significant effects of COS, PRO or their combination (CPRO) on growth performance and incidences and severity of diarrhea as observed in our results.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

During the pre-challenge period, 0.4% SDWE-fed piglets had greater ADG and G:F but not ADFI compared to the control whereas during the post-*E. coli* challenge, growth performance was not affected by dietary treatment. Also, the intestinal histomorphology (VH, CD & VH:CD) was not different among treatments and although SDWE-fed piglets recovered faster from diarrhea than the control, the differences were not significant. Supplementation of pig starter diet with 400 mg/kg COS or 100 mg/kg *Enterococcus fecalis* probiotic (PRO) or their combination (CPRO) did not significantly affect any of the growth performance parameters or reduce ETEC K88-induced diarrhea in 21-d-old piglets before and after challenge. It can, therefore, be concluded based on the results of the present research that: 1) growth-promoting effects of spray-dried whole egg powder containing anti-F4 antibodies (SDWE) produced against recombinant ETEC F4 fimbrial antigens appeared to be dose-dependent, especially as observed during the pre-challenge period; 2) 0.4% SDWE (SDWE2) supported greater growth performance before challenge but such benefit was not evident during the post-challenge period with either 0.1% or 0.4% inclusion level of SDWE; 3) chitosan oligosaccharide (COS) or *Enterococcus fecalis* probiotic or their combination (CPRO) did not significantly reduce ETEC $K88^+$ induced diarrhea in early-weaned piglets; and 4) COS or *Enterococcus fecalis* probiotic or their combination had no significant effects on improving growth performance in ETEC $K88^+$ challenged piglets. Further research is suggested to:

1. Validate the effective dose of SDWE using various dosages including those used in the present study and higher doses taking into consideration some cost implications when compared with AGP use.

2. Determine effects of gastric juice and digestive enzymes on SDWE. This will help find means of protecting the antibody to reach target sites such as jejunum and ileum.
3. Investigate the underlying mechanism of growth-promoting property of SDWE.
4. Determine antibacterial effects of chitosan on Gram-positive bacteria especially those used as probiotics including lactic acid-producing bacteria e. g. *Enterococcus fecalis*. The aim of this will be to determine if there is synergy between chitosan and probiotics as effective and viable alternative means to AGPs.
5. Determine the enhanced effect of supplementing piglet diet containing chitosan and chicken egg antibodies combination on growth performance and gut health in early-weaned piglets.

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