Animal Nutrition 3 (2017) 366–371

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Combined effects of chitosan and microencapsulated *Enterococcus faecalis* CG1.0007 probiotic supplementation on performance and diarrhea incidences in enterotoxigenic *Escherichia coli* K88$^+$ challenged piglets

Kolawole Aluko [a], Deepak E. Velayudhan [a], Ehsan Khafipour [a], Aike Li [b], Yulong Yin [c], Martin Nyachoti [a, *]

[a] Department of Animal Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada
[b] Academy of Science and Technology of State Administration of Grain, Beijing 100037, China
[c] Institute of Subtropical Agriculture, Chinese Academic of Sciences, Changsha 410125, China

ARTICLE INFO

Article history:
Received 27 January 2017
Received in revised form
22 August 2017
Accepted 5 September 2017
Available online 21 September 2017

Keywords:
Enterotoxigenic *Escherichia coli* K88$^+$
Chitosan oligosaccharide
Enterococcus faecalis CG1.0007 probiotic
Growth performance
Diarrhea incidences
Piglets

ABSTRACT

The aim of this study was to investigate the combined effects of chitosan oligosaccharide (COS) and a microencapsulated *Enterococcus faecalis* CG1.0007 probiotic (PRO) on growth performance and diarrhea incidences in enterotoxigenic *Escherichia coli* (ETEC) K88$^+$ challenged piglets in a 14-d study. Thirty piglets, 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), 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). 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 (1×10^{11} cfu/mL) of freshly grown ETEC inoculum. During post-challenge period, blood was sampled at 24 and 48 h to determine plasma urea nitrogen (PUN), and diarrhea incidences and fecal consistency scores were recorded from d 9 to 12. On d 14, all pigs were weighed and then euthanized to obtain intestinal tissue samples for histomorphometric measurements. Growth performance responses were 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 = 0.0001) villus:crypt ratio compared with the NC. Under the conditions of the present study, it can be concluded that supplementation of piglet diets with 400 mg/kg COS, 100 mg/kg microencapsulated PRO or their combination did not significantly improve piglet growth performance both during the pre- and post-ETEC K88$^+$ oral inoculation. Also, there were no significant reduction of incidences and severity of diarrhea after challenge compared with the control group.

1. Introduction

Infection with enterotoxigenic *Escherichia coli* (ETEC) expressing K88$^+$ (F4) fimbriae is one of the most important causes of post weaning diarrhea in pigs with significant economic losses (Fairbrother et al., 2005; Daudelin et al., 2011). These losses result 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.,

* Corresponding author.
E-mail address: Martin.Nyachoti@umanitoba.ca (M. Nyachoti).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2017.09.003

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 antimicrobial growth promoters (AGP).

In animal agriculture, antimicrobials are used not only for growth promotion in sub-therapeutic doses, but also for disease prevention (prophylactic doses) and treatment (therapeutic doses) (Diraviyam et al., 2014). Moreover, many reports have demonstrated the significant contributions of antimicrobials to the improved performance of animals (Turner et al., 2001; Cromwell, 2002). However, there are concerns about antimicrobial usage due to antimicrobial drug residues in food animal products and increased antibiotic resistant bacteria (Diraviyam et al., 2014). As a result, there is increased public pressure to eliminate the use of in-feed antibiotics as AGP in livestock diets (Hulst et al., 2013), hence the need for identifying effective and viable alternative therapies to AGP (Owusu-Asiedu et al., 2003; Kiarie et al., 2009, 2011). Any replacement for AGP 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 such alternative therapy is a combination of chitosan oligosaccharide (COS) and *Enterococcus faecalis* CG1.0007 probiotic (PRO) because of a possible synergy of actions. Also, enhanced effects between these 2 additives are expected 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 (Haixiang et al., 2005). Chitosan supplementation has been shown to have inhibitory effects on *E. coli* in piglets by reducing the incidence of diarrhea and dependence on antimicrobials (Haixiang et al., 2005). It has also been reported to improve growth performance and nutrient digestibility in weaned piglets (Xu et al., 2014). Being a polycationic molecule (Rabea et al., 2003), chitosan can bind to the predominantly anionic cell surface of Gram-negative bacteria such as *E. coli.* This binding results in changes in the outer membrane permeability and subsequent leakage of cell constituents such as enzymes and glucose (Rabea et al., 2003), thus, preventing its growth and spread of *E. coli*. Moreover, this would render *E. coli* more sensitive to the inhibitory action of bile and organic acids such as lactic acid produced by probiotic bacteria in the class of lactic acid-producing bacteria (LAB) including *Lactobacilli*, *Enterococci* and *Bifidobacteria* (Brocklehurst and Lund, 1990; Bednorz et al., 2013). 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 gastrointestinal tract. 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 short chain 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 this 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.

2. 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).

2.1. Animals, treatments and oral challenge

Thirty piglets ([Yorkshire × Landrace] × 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: 76 cm × 61 cm × 38 cm) 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 °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), 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, China), NC + 100 mg/kg (1×10^9 cfu/kg) PRO (PRO; SKF Biotechnology Company Ltd., Beijing, China) and NC + a combination of COS and PRO (CPRO). The basal diet (Table 1) was formulated to meet the NRC (2012) nutrient

Table 1
Composition and calculated nutrient levels of basal diet (as-fed basis).

Item	Content
Ingredients, %	
Corn	14.35
Wheat	30.00
Soybean meal	28.00
Dried whey	19.00
Vegetable oil	5.00
Limestone	0.77
$CaHPO_4$	0.76
Iodized salt (NaCl)	0.42
Vitamin–mineral premix[1]	1.00
Lysine-HCl	0.33
DL-methionine	0.20
Threonine	0.14
Tryptophan	0.30
Calculated nutrient levels, %	
ME, MJ/kg	14.4
Crude protein	20.84
Lysine	1.49
Methionine	0.50
Methionine + Cysteine	0.87
Threonine	0.95
Tryptophan	0.30
Calcium	0.78
Total phosphorus	0.50
Analyzed nutrient levels, %	
Dry matter	89.9
Gross energy, MJ/kg	18.4
Crude protein	21.31
Calcium	0.81
Total phosphorus	0.56

[1] Vitamin-premix provided per kg of diet: vitamin A 8,250 IU, vitamin D_3 835 IU, vitamin E 40 IU, vitamin K_3 4 mg, vitamin B_{12} 0.025 mg, vitamin B_1 2 mg, vitamin B_2 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.

specifications for 5 to 10 kg BW pigs. Feed and water were provided *ad libitum*. 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. Subsequently, each pig was orally challenged with 6 mL (1×10^{11} cfu/mL) of freshly grown ETEC inoculum. Body weight and feed intake 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 2 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 and a score of $\leq$1 was considered to indicate no diarrhea.

2.2. Isolation, identification, and microencapsulation of probiotic strain

The microencapsulated PRO were prepared and obtained from the Academy of State Administration of Grain, Beijing, China (Han et al., 2013). In brief, a total of 63 potentially probiotic strains were obtained from intestinal enterococci of human infants (CG1.0022-24, and CG1.0036), pigs (CG1.0001, CG1.0003, and CG1.0005), and broilers (CG1.0006-7, CG1.0010, CG1.0013, and CG1.0015) by selective culturing. Thirteen of these strains were identified as *Enterococcus faecium* or *Enterococcus fecalis* by Biolog automated microanalysis system (Biolog) and 16S rRNA gene sequencing. *E. fecalis* strain CG1.0007 (from broilers) was selected and isolated on the basis of growth and metabolic performance, inhibition of pathogenic bacteria, and resistance to adverse conditions. This strain displayed rapid growth (generation time approximately 20 min), short lag phase (1.5 h), high L-lactic acid yield (up to 8 g/L), and the ability to inhibit common pathogenic bacteria.

2.3. ETEC K88$^+$ and culture condition

The ETEC K88$^+$ strain was originally obtained from Veterinary Diagnostic Services of Manitoba, Winnipeg, Manitoba, Canada. From the frozen stock, ETEC K88$^+$ was streaked on brain heart infusion (BHI) agar and grown anaerobically at 37 °C overnight. Then a single colony was inoculated on 2 BHI plates (i.e., duplicate) and incubated anaerobically at 37 °C overnight. Two tubes of 5 mL BHI (BD & Co., Franklin Lakes, New Jersey, USA) broth plus 2% casamino acids (Fisher Scientific, Waltham, MA, USA) were inoculated from a single colony and grown overnight at 37 °C with shaking (200 rpm). The ETEC K88$^+$ identity was verified using an ETEC 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 °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 phosphate buffered saline (PBS), 10^6 to 10^9 dilutions were plated on BHI plates to check that the culture was $>1 \times 10^9$. Incubation was done anaerobically at 37 °C overnight. The colonies on the dilution plates were counted the following day to determine concentration. For inoculation, 6 mL of 1×10^{11} cfu/mL per piglet were used.

2.4. Blood sample collections

Heparinized blood samples to obtain plasma for PUN concentration analysis were collected from the jugular vein of each piglet on d 8 before *E. coli* oral inoculation, at 24 and 48 h post *E. coli* challenge (d 9 and 10 of study). Samples were immediately centrifuged at 3,000 $\times$ *g* for 15 min at 5 °C to harvest plasma and stored at −20 °C until required for PUN analysis.

2.5. Intestinal tissue collection

Ileal sections were collected from all the piglets on d 7 post ETEC challenge after being anesthetized by an intramuscular injection of ketamine:xylazine (20 mg/kg:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and euthanized by an intracardiac injection of sodium pentobarbital (50 mg/kg of BW; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada). Fifteen centimeters cranial to 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 10 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 depth of the crypt from this junction to the base and villus-crypt ratio (VH:CD) determined.

2.6. Sample preparation and chemical analyses

Experimental diets were ground to pass through a 1 mm screen before chemical analysis. Feed samples were analyzed for dry matter, crude protein, gross energy, Ca and P. Dry matter content was determined according to the AOAC (1990) by oven drying 5 g of sample at 102 °C overnight. Gross energy was measured using an adiabatic bomb calorimeter (model 6400, Parr Instrument, Moline, IL, USA) which had been calibrated using benzoic acid as a standard. Nitrogen content was determined by the combustion method (AOAC, 2005) using the LECO N analyser (model CNS-2000; LECO Corp., St. Joseph, MI, USA) and crude protein was calculated as nitrogen $\times$ 6.25. Samples for analysis of Ca and P were ashed for 12 h and digested according to AOAC (2005) and read on a Varian inductively coupled plasma mass spectrometer (Varian Inc., Palo Alto, CA, USA).

2.7. Statistical analysis

Data obtained 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 NC, PC, COS, PRO and CPRO) were the fixed effects. Means for significant treatment differences were compared by the least significant difference (LSD) test. Chi-square test was performed on diarrhea incidence to determine if differences among the treatment groups were significant. Probability values of $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. Growth performance

The effects of dietary treatment on ADG, ADFI and G:F are presented in Table 2. Final BW was similar among treatments and dietary supplementation with COS, PRO or CPRO did not significantly

Table 2
Growth performance of piglets fed chitosan oligosaccharide (COS), probiotic and a combination of COS and probiotic.

Item	Treatments[1]						
	NC	PC	COS	PRO	CPRO	SEM	*P*-value
IBW, kg	7.21	7.12	7.21	7.23	7.20	0.228	0.999
FBW, kg	9.94	10.38	9.48	10.30	9.24	0.484	0.521
ADG, g/d							
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/d							
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

IBW = initial body weight; FBW = final body weight; ADG = average daily gain; SEM = standard error of the mean; ADFI = average daily feed intake; G:F = the ratio of BW gain to feed intake.

[1] NC = negative control; PC = positive control, NC + 2.5 g chlortetracycline/kg feed; COS = NC + 400 mg COS/kg feed (inclusion rate of COS was determined based on manufacturer's recommendations and previous studies such as Xu et al., 2013; Xu et al., 2014; Xiao et al., 2014); PRO = NC + 100 mg *Enterococcus faecalis* CG1.0007 probiotic (1.0×10^{10} cfu/g microcapsules)/kg feed (inclusion rate of probiotic was determined based on manufacturer's recommendations and previous studies such as Han et al., 2013); CPRO = NC + 400 mg COS/kg feed and 100 mg *Enterococcus faecalis* CG1.0007 probiotic (microcapsules)/kg feed.

affect the growth performance both during the pre- and post-ETEC K88$^+$ challenge periods.

3.2. Plasma urea nitrogen

There were no differences in PUN concentrations among dietary treatments before challenge and at 24 and 48 h after ETEC K88$^+$ challenge. However, there was a numerical increase in PUN concentrations from baseline values at 24 h post inoculation irrespective of dietary treatment (Table 3).

3.3. Diarrhea

Diarrhea (Table 4) was not observed during the pre-challenge period. However, 12 h post-ETEC K88$^+$ oral inoculation, 2 (33%) piglets each from the NC and PRO-fed pigs developed mild diarrhea, while one piglet (17%) each from those fed the COS and CPRO diets had diarrhea (Table 3). Piglets fed the PC diet did not develop diarrhea. One-day post-challenge, 4 (67%) piglets in the NC had severe diarrhea, whereas 3 (50%) in CPRO, 2 (33%) in PRO and 1 (17%) each in PC and COS-fed groups had mild diarrhea. Three days post-challenge, 3 (50%) piglets in the NC group had severe diarrhea, whereas 1 (17%) each from the PRO and CPRO groups had mild diarrhea; none of the piglets in the PC and COS groups had diarrhea. These observed incidences and severity of diarrhea were not statistically different among the 5 dietary treatments ($P > 0.10$).

Table 3
Plasma urea nitrogen (PUN) content and ileal histomorphology of piglets fed chitosan oligosaccharide (COS), probiotic and a combination of COS and probiotic.

Item	Treatments[1]						
	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	0.526
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	0.099
CD, μm	262.5	206.7	260.0	254.0	247.5	17.22	0.253
VH:CD	1.07^b	1.76^a	1.28^b	1.24^b	1.25^b	0.09	0.001

VH = villus height; CD = crypt depth; VH:CD = villus: crypt ratio; SEM = standard error of the mean.

^{a,b} Values within a row with different letters are significantly different ($P < 0.05$).

[1] NC = negative control; PC = positive control, NC + 2.5 g chlortetracycline/kg feed; COS = NC + 400 mg COS/kg feed (inclusion rate of COS was determined based on manufacturer's recommendations and previous studies such as Xu et al., 2013; Xu et al., 2014; Xiao et al., 2014); PRO = NC + 100 mg *Enterococcus faecalis* CG1.0007 probiotic (1.0×10^{10} cfu/g microcapsules)/kg feed (inclusion rate of probiotic was determined based on manufacturer's recommendations and previous studies such as Han et al., 2013); CPRO = NC + 400 mg COS/kg feed and 100 mg *Enterococcus faecalis* CG1.0007 probiotic (microcapsules)/kg feed.

Table 4
Diarrhea incidences in piglets fed chitosan oligosaccharide (COS), probiotic and a combination of COS and probiotic.[1]

Post-challenge	Treatments[2]					
	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] The data were represented as number of piglets with diarrhea/total (fecal consistency [FC] score). The FC for each piglet was determined by averaging the assigned scores: 0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea. Fecal consistency score ≤1 means no diarrhea.

[2] NC = negative control; PC = positive control, NC + 2.5 g chlortetracycline/kg feed; COS = NC + 400 mg COS/kg feed (inclusion rate of COS was determined based on manufacturer's recommendations and previous studies such as Xu et al., 2013; Xu et al., 2014; Xiao et al., 2014); PRO = NC + 100 mg *Enterococcus faecalis* CG1.0007 probiotic (1.0×10^{10} cfu/g microcapsules)/kg feed (inclusion rate of probiotic was determined based on manufacturer's recommendations and previous studies such as Han et al., 2013); CPRO = NC + 400 mg COS/kg feed and 100 mg *Enterococcus faecalis* CG1.0007 probiotic (microcapsules)/kg feed.

3.4. Ileal histomorphology

Villi height tended ($P = 0.099$) to differ among dietary treatments but there no differences in CD ($P = 0.253$). The VH:CD was significantly affected ($P = 0.0001$) by dietary treatment (Table 3).

4. Discussion

The ETEC K88$^+$ oral challenge model used in the present study was insufficiently sensitive to produce responses in challenged piglets similar to clinical cases of post-weaning colibacillosis. Final BW was similar among treatments and dietary supplementation with COS, PRO or CPRO had similar effects on growth performance during both the pre- and post-ETEC K88$^+$ challenge periods. However, the observation that piglets fed the CPRO diet had the highest G:F that was also significantly different from that of the piglets fed the NC diet suggests that combining COS with PRO may improve the efficiency of nutrient utilization. Although the effects of chitosan on growth performance of broilers, pigs or other livestock species are not consistent (Xu et al., 2014), its dietary supplementation has been shown to support superior growth performance and FCR in broilers (Suk, 2004; Khambualai et al., 2008, 2009) and improved growth rates in pigs (Tang et al., 2005; Walsh et al., 2013; Xiao et al., 2014; Xu et al., 2014). The positive effects of chitosan supplementation in pigs have been 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). It should be noted, however, that contradictory observations have been reported regarding the effects of dietary chitosan supplementation in poultry and pigs. For example, Razdan et al. (1997) observed significant reductions in body weight and feed intake of broiler chickens fed 30 g/kg chitosan compared with those fed the control diet. Similarly, non-

positive effects on nutrient digestibility in pigs have been reported (Razdan and Patterson, 1994, 1996; O'Shea et al., 2011).

The positive gut health effects of chitosan may be linked to its antimicrobial activity against pathogenic microorganisms particularly the Gram-negative bacteria such as *E. coli*. Chitosan electrostatically interacts with bacterial cell wall and membrane (Rabea et al., 2003; Kong et al., 2010). This causes loss of cell wall protection and exposure of cell membrane leading to a drastic increase in membrane permeability and eventually cell death (Kong et al., 2008, 2010). Thus, in the present study it was anticipated that dietary COS supplementation will confer similar gut health benefits in ETEC challenged pigs. However, based on the findings of the present study, this was not the case during either the pre- or post-challenge period. This observation may be attributed to the observed reduction in feed intake. Yuan and Chen (2012) reported that dietary chitosan supplementation improved growth performance of young pigs by increasing feed intake. The observed similar effects on growth performance in CPRO-fed piglets compared with COS or PRO group can partly be explained by some antibacterial activity of chitosan on Gram-positive bacteria such as *E. faecalis* (used as probiotic in the present study). Nevertheless, the inhibitory effect has been reported to be more pronounced on Gram-negative bacteria because of the possession of higher negative charges (polyanions) on cell surface and adsorption of more chitosan on to the Gram-negative bacterial cell wall (Chung et al., 2004). The cell wall of Gram-positive 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).

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 vs. 105 g/d; ADFI = 208 vs. 162 g/d; G:F = 0.86 vs. 0.59; post-challenge: ADG = 350 vs. 291 g/d; ADFI = 449 vs. 348 g/d; 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.

A closer look at the performance data obtained in the current study reveals that feeding chitosan containing diets (COS and CPRO) had a negative effect on feed intake during the pre-challenge period (24.3% and 35.1% reduction for COS and CPRO groups, respectively). This observation was also noted during the post-challenge period (19.3% and 11.4% reduction for COS and CPRO groups, respectively). The reduction in ADFI could explain the growth rate data showing considerable reduction in ADG during the pre-challenge period. During post-challenge, feeding diets containing probiotic or the combination of chitosan and probiotic supported ADG that was approximately 17% higher than that obtained with NC diet and similar to the level produced by PC diet. It is possible that these treatment effects were not statistically significant due to the smaller sample size used in the present study and the fact that piglets were housed individually, which tends to increase variability among replicates. Therefore, further studies will be required to elucidate the potential benefits of the additives evaluated in the present study in terms of piglet performance. Furthermore, the indication that COS may adversely affect feed consumption in piglets should be examined in larger studies with group housed piglets.

The PUN results demonstrated that no significant effects were observed before challenge and at 24 and 48 h after ETEC K88$^+$ 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 K88$^+$ on energy metabolism as a result of inefficient utilization of dietary protein and body protein breakdown for synthesis of acute phase proteins in the hepatocytes (Coma et al., 1995). Though not significant, the increase in PUN levels following ETEC K88$^+$ challenge also provides further evidence of a mild infection achieved in this study. Previous studies have demonstrated that ETEC infection increases PUN concentration in piglets (Owusu-Asiedu et al., 2003). The lack of significant treatment effects could be attributed to the failure of the challenge model to produce responses similar to clinical cases of ETEC K88$^+$ infection in piglets. However, the numerical differences in PUN concentrations among treatments show slightly higher values for piglets fed COS-containing diets.

Similarly, the results of dietary treatment effects on incidences and severity of diarrhea indicated no significant differences. From these observations, piglets fed NC and CPRO had the highest incidences and severity of diarrhea compared with PC, COS and PRO fed piglets. Hence, the observation supports the growth performance results and suggests no significant synergistic effect of CPRO in reducing ETEC-induced diarrhea in early-weaned piglets.

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 (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 probably resulting from crypt hyperplasia for the repopulation of epithelial cells (Zhang and Xu, 2003; Llyod and Gabe, 2008). However, contrary to previous reports (Walsh et al., 2013; Liu et al., 2008) that improved intestinal structure significantly promotes growth performance, no significant growth improvement was observed in our data. These discrepancies may be attributable to different experimental designs and methodologies, age and breed of animals, genetic factors, types and dosages of additives and anti-nutritive factors in diets.

5. Conclusion

Under the conditions of the present study, it can be concluded that supplementation of piglet diets with 400 mg/kg COS, 100 mg/kg microencapsulated PRO or their combination did not significantly support improved piglet growth performance both during the pre- and post-ETEC K88$^+$ oral inoculation. Similarly, no reduction of incidences and severity of diarrhea were observed after challenge in early-weaned piglets compared with the control group. However, the results of the present study can be applied for collecting big data to predict supplemental chitosan amount and its combinations.

Acknowledgments

This project was funded by Natural Sciences and Engineering Research Council of Canada and Manitoba Pork Council. The technical assistance from our lab members for sampling and analyses, Hein Min Tun and Robert Stuski for helping with ETEC K88$^+$ inoculum and animal care, respectively, are gratefully acknowledged.

References

Association of Official Analytical Chemists. Official methods of analysis. 15th ed. Washington, DC: AOAC; 1990.

Association of Official Analytical Chemists. Official methods of analysis. 18th ed. Arlington, VA: AOAC; 2005.

Bednorz C, Guenther S, Oelgeschager K, Kinnemam B, Pieper R, Hartmann S, et al. Feeding the probiotic *Enterococcus faecium* strain NCIMB 10415 to piglets specifically reduces the number of *Escherichia coli* pathotypes that adhere to the gut mucosa. Appl Environ Microbiol 2013;79:7896–904.

Boudry G, Lalles JP, Malbert CH, Bobillier E, Steve B. Diet-related adaptation of the small intestine at weaning in pigs is functional rather than structural. J Pediatr Gastroenterol Nutr 2002;34:180–7.

Boudry G, Guerin S, Malbert CH. Effect of an abrupt switch from a milk-based to a fibre-based diet on gastric emptying rates in pigs: difference between origins of fibres. Br J Nutr 2004;92:913–20.

Brocklehurst TF, Lund BM. The influence of pH, temperature and organic acids on the initiation of growth of *Yersinia enterocolitica*. J Appl Bacteriol 1990;69:390–7.

Canadian Council of Animal Care. Guide to the care and use of experimental animals. 2nd ed., vol. 1. Ottawa, ON: CCAC; 2009.

Cera KR, Mahan DC, Cross RF, Reinhart GA, Whitmoyer RE. Effect of age, weaning, and post-weaning diet on small intestinal growth and jejunal morphology in young swine. J Anim Sci 1988;66:574–84.

Chen YJ, Kim IH, Cho JH, Yoo JS, Wang Y, Huang Y, et al. Effects of chitooligosaccharide supplementation on growth performance, nutrient digestibility, blood characteristics and immune responses after lipopolysaccharide challenge in weanling pigs. Livest Sci 2009;124:255–60.

Chung YC, Su YP, Chen CC, Jia J, Wang HL, Wu JCG, et al. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. Acta Pharmacol Sin 2004;25:932–6.

Coma J, Carrion D, Zimmerman DR. Use of plasma urea nitrogen as a rapid response criterion to determine the lysine requirements of pigs. J Anim Sci 1995;73:472–81.

Cromwell GL. Why and how antibiotics are used in swine production. Anim Biotechnol 2002;13:7–27.

Daudelin JF, Lessard M, Beaudoin F, Nadeau E, Bissonnette N, Boutin Y, et al. Administration of probiotics influences F4 (K88)-positive enterotoxigenic *Escherichia coli* attachment and intestinal cytokine expression in weaned pigs. Vet Res 2011;42:69–79.

Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. Poult Sci 2005;84:634–43.

Diraviyam T, Zhao B, Wang Y, Schade R, Michael A, Zhang X. Effect of chicken egg yolk antibodies (IgY) against diarrhea in domesticated animals: a systematic review and meta-analysis. PLoS One 2014;9(5):e97716. 1–14.

Fairbrother JM, Nadeau E, Gyles CL. *Escherichia coli* in post weaning diarrhea in pigs: an update on bacterial types, pathogenesis and prevention strategies. Anim Health Res Rev 2005;6:17–39.

Haixiang S, BaoQiang S, Mersheng X. Inhibitory effects of different molecular weight chitosan on *Escherichia coli* (K88). Chin J Anim Sci 2005;41:30–1.

Han W, Zhang XL, Wang DW, Li LY, Liu GL, Li AK, et al. Effects of microencapsulated *Enterococcus fecalis* CG1.0007 on growth performance, anti-oxidation activity, and intestinal microbiota in broiler chickens. J Anim Sci 2013;91:4374–82.

Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol 2001;71:235–44.

Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr 2013;97:207–37.

Hornich M, Salajka L, Ulmann Z, Sedlacek M. Enteric *Escherichia coli* infections. Vet Pathol 1973;10:484–500.

Hulst M, Vastenhouw S, Smits M, de Wit A, Niewold T, van der Meulen J. Transcription networks responsible for early regulation of *Salmonella*-induced inflammation in the jejunum of pigs. J Inflamm 2013;10:1–15.

Khambualai O, Yamauchi K, Tangtaweewipat S, Cheva-Isarakul B. Effects of dietary chitosan diets on growth performance in broiler chickens. J Poult Sci 2008;45:206–9.

Khambualai O, Yamauchi K, Tangtaweewipat S, Cheva-Isarakul B. Growth performance and intestinal histology in broiler chickens fed with dietary chitosan. Br Poult Sci 2009;50:592–7.

Kiarie E, Slominski BA, Krause DO, Nyachoti CM. Acute phase response of piglets fed diets containing non-starch polysaccharide hydrolysis products and egg yolk antibodies following an oral challenge with *Escherichia coli* (K88). Can J Anim Sci 2009;89:353–60.

Kiarie E, Bhandari S, Scott M, Krause DO, Nyachoti CM. Growth performance and gastrointestinal microbial ecology responses of piglets receiving *Saccharomyces cerevisiae* fermentation products after an oral challenge with *Escherichia coli* (K88). J Anim Sci 2011;89:1062–78.

Kong M, Chen XG, Liu CS, Liu CG, Meng XH, Yu LJ. Antibacterial mechanism of chitosan microspheres in a solid dispersing system against *E. coli*. Colloids Surf B Biointerfaces 2008;65:197–202.

Kong M, Chen XG, Xing K, Park HJ. Antimicrobial properties of chitosan and mode of action: a state of the art review. Int J Food Microbiol 2010;144:51–63.

Lim HS, Paik IK, Sohn TI, Kim WY. Effects of supplementary copper chelates in the form of methionine, chitosan and yeast on the performance of broilers. Asian-Australas J Anim Sci 2006;19:1322–7.

Liu P, Piao XS, Kim SW, Wang L, Shen YB, Lee HS, et al. Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology and fecal shedding of *Escherichia coli* and *Lactobacillus* in weaning pigs. J Anim Sci 2008;86:2609–18.

Llyod DAJ, Gabe SM. Intestinal morphology, intestinal regeneration and the promise of tissue engineering. In: Langnas AN, Goulet O, Quigley EMM, Tappenden KA, editors. Intestinal failure: diagnosis, management and transplantation. Maiden, MA, USA: Wiley-Blackwell; 2008. p. 13.

Madec F, Bridoux N, Bounaix S, Cariolet R, Duval-Iflah Y, Hampson DJ, et al. Experimental models of porcine post-weaning colibacillosis and their relationship to post-weaning diarrhea and digestive disorders as encountered in the field. Vet Microbiol 2000;72:295–310.

Marquardt RR, Jin LZ, Kim JW, Fang L, Frohlich AA, Baidoo SK. Passive protective effect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88+ infection in neonatal and early-weaned piglets. FEMS Immunol Med Microbiol 1999;23:283–8.

McCracken BA, Spurlock ME, Roos MA, Zuckermann FA, Gaskins HR. Weaning anorexia may contribute to local inflammation in the piglet small intestine. J Nutr 1999;129:613–9.

Moeser AJ, Ryan KA, Nighot PK, Blikslager AT. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. Am J Physiol Gastrointest Liver Physiol 2007;293:413–21.

National Research Council. Nutrient requirements of swine. 11th ed. NRC: National Academy of Press; 2012.

O'Shea CJ, Sweeney T, Lynch MB, Callan JJ, O'Doherty JV. Modification of selected bacteria and markers of protein fermentation in the distal gastrointestinal tract of pigs upon consumption of chitosan is accompanied by heightened manure odor emissions. J Anim Sci 2011;89:1366–75.

Owusu-Asiedu A, Nyachoti CM, Marquardt RR. Response of early-weaned pigs to an enterotoxigenic *Escherichia coli* (K88) challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid, or antibiotic. J Anim Sci 2003;81:1790–8.

Pluske JR, Hampson DJ, Williams IH. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest Prod Sci 1997;51:215–36.

Raafat D, Bargen KV, Haas A, Sahl HG. Insights into the mode of action of chitosan as an antibacterial compound. Appl Environ Microbiol 2008;74:3764–73.

Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W. Chitosan as antimicrobial agent: applications and mode action. Biomacromolecules 2003;4:1457–65.

Razdan A, Patterson D. Effect of chitin and chitosan on nutrient digestibility and plasma lipid concentrations in broiler chickens. Br J Nutr 1994;72:277–88.

Razdan A, Patterson D. Hypolipidaemic, gastrointestinal and related responses of broiler chickens to chitosans of different viscosity. Br J Nutr 1996;76:387–97.

Razdan A, Patterson D, Patterson J. Broiler chicken body weights, feed intakes, plasma lipid and small intestinal bile acid concentrations in response to feeding of chitosan and pectin. Br J Nutr 1997;78:283–91.

Stein HH, Kil DY. Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, part 2. Anim Biotechnol 2006;17:217–31.

Suk YO. Interaction of breed-by-chitosan supplementation on growth and feed intake efficiency at different supplementing ages in broiler chickens. Asian-Australas J Anim Sci 2004;17:1705–11.

Tang ZR, Yin YL, Nyachoti CM, Huang RL, Li TJ, Yang CB, et al. Effect of dietary supplementation of chitosan and galacto-mannan-oligosaccharide on serum parameters and the insulin-like growth factor-1 mRNA expression in early-weaned piglets. Domest Anim Endocrinol 2005;28:430–41.

Turner J, Dritz S, Minton J. Review: alternative to conventional antimicrobials in swine diets. Prof Anim Sci 2001;17:217–26.

Walsh AM, Sweeney T, Bahar B, O'Doharty VJ. Multi-functional roles of chitosan as a potential protective agent against obesity. PLoS One 2013;8:1–7.

Xiao D, Wang Y, Liu G, He J, Qiu W, Hu X, et al. Effects of chitosan on intestinal inflammation in weaned pigs challenged by enterotoxigenic *Escherichia coli*. PLoS One 2014;9(8):e104192. 1-7.

Xu Y, Shi B, Yan S, Li T, Guo Y, Li J. Effects of chitosan on body weight gain, growth hormone and intestinal morphology in weaned pigs. Asian-Australas J Anim Sci 2013;10:1484–9.

Xu Y, Shi B, Yan S, Li J, Guo Y, Guo X. Effects of chitosan supplementation on the growth performance, nutrient digestibility, and digestive enzymes activity in weaned pigs. Czech J Anim Sci 2014;59:156–63.

Yokoyama H, Peralta RC, Diaz R, Sendo S, Ikemori Y, Kodama Y. Passive effect of chicken egg-yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli*. Infect Immun 1992;60:998–1007.

Yuan SB, Chen H. Effects of dietary supplementation of chitosan on growth performance and immune index in ducks. Afr J Biotechnol 2012;11:3490–5.

Zhang Y, Xu RJ. Anatomy and histology of the gastrointestinal tract. In: Xu RJ, Cranwell PD, editors. The neonatal pig: gastrointestinal Physiology and nutrition. Thrumpton, Nottingham, UK: Nottingham University Press; 2003. p. 1.