Combined effects of chitosan and microencapsulated Enterococcus faecalis CG1.0007 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 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 (\(P = 0.001\)) 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.

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**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., 2007).
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 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, 2003). 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 (Rabeta 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 (Rabeta 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 (Rong 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 Enterobacteriacaee (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% chlorotetracycline (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 × 10⁹ cfu/kg) PRO (PRO; SKF Biotechnology Company Ltd., Beijing, China) and NC + a combination of COS and PRO (CPR). The basal diet (Table 1) was formulated to meet the NRC (2012) nutrient requirements of pigs. 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).

| Item                          | Content |
|-------------------------------|---------|
| Ingredients, %                |         |
| Corn                          | 14.35   |
| Wheat                         | 3.00    |
| Soybean meal                  | 2.80    |
| Dried whey                    | 1.90    |
| Vegetable oil                 | 5.00    |
| Limestone                     | 0.77    |
| CaHPO4                        | 0.76    |
| Iodized salt (NaCl)           | 0.42    |
| Vitamin–mineral premix¹       | 1.00    |
| Lysine–HCl                    | 0.33    |
| α-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 D3 835 IU, vitamin E 40 IU, vitamin K3 4 mg, vitamin B12 0.025 mg, vitamin B2 2 mg, vitamin B6 12 mg, nicotinic acid 22.5 mg, folic acid 2 mg, pyridoxine 4.5 mg, biotin 0.2 mg, pantotthenic 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 ≤ 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 faecalis by Biolog automated micro-analyses system (Biolog) and 16S rRNA gene sequencing. E. faecalis 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 with shaking (200 rpm). The ETEC K88⁻ strain was identified using an ETEC K88⁻ fimbrex latex agglutination kit. Two flasks of 500 mL BHI 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), 10⁻² to 10⁻⁶ dilutions were plated on BHI plates to check that the culture was >1 × 10⁶. 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 × 10¹¹ 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 × 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; Bimed-MTC Animal Health Inc., Cambridge, Ontario, Canada) and euthanized by an intracardiac injection of sodium pentobarbital (50 mg/kg of BW; Bimed-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 Image 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 a 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 × 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 (fixed 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 ≤ 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
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).

### 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 PC 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; Khambula 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., 2008), 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-

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**Table 2**

| Item          | Treatments | P-value |
|---------------|------------|---------|
| IBW, kg      | NC 7.21 PC 7.12 COS 7.21 PRO 7.23 CPRO 7.20 | 0.228 0.999 |
| FBW, kg      | NC 9.94 PC 10.38 COS 9.84 PRO 10.30 CPRO 9.24 | 0.484 0.521 |
| ADG, g/d     | NC 156 ADG 176 PC 105 COS 162 PRO 97 | 0.406 0.547 |
| ADFI, g/d    | NC 299 ADFI 367 PC 291 COS 350 PRO 353 CPRO 340 | 0.477 0.577 |
| G:F          | NC 0.62 PC 0.78 COS 0.59 PRO 0.86 CPRO 0.61 | 0.12 0.375 |

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.

| Item        | Treatments | P-value |
|-------------|------------|---------|
| Plasma urea nitrogen (PUN) content and ileal histomorphology of piglets fed chitosan oligosaccharide (COS), probiotic and a combination of COS and probiotic. | | |
| Item        | Treatments | P-value |
|-------------|------------|---------|
| Villi height (VH) | NC 1.92 PC 1.58 COS 1.10 PRO 1.77 CPRO 1.30 | 0.33 0.526 |
| Crypt depth (CD) | NC 2.97 PC 2.23 COS 3.22 PRO 2.38 CPRO 3.23 | 0.46 0.530 |
| VH:CD ratio | NC 2.87 PC 2.07 COS 2.62 PRO 2.37 CPRO 2.55 | 0.46 0.821 |
| VH/CD       | NC 283.8 PC 359.7 COS 320.3 PRO 310.3 CPRO 301.0 | 17.72 0.099 |
| VH:CD       | NC 262.5 PC 206.7 COS 260.0 PRO 254.0 CPRO 247.5 | 17.22 0.253 |

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

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**Table 3**

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

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**Table 4**

| Item        | Treatments | P-value |
|-------------|------------|---------|
| Diarrhea incidences in piglets fed chitosan oligosaccharide (COS), probiotic and a combination of COS and probiotic. | | |
| Post-challenge | Treatments | P-value |
|-------------|------------|---------|
| 12 h        | NC 2/6 (2.0) PC 0/6 (0.0) COS 1/6 (2.0) PRO 2/6 (2.0) CPRO 1/6 (2.0) | 0.549 |
| d 1         | NC 4/6 (2.5) PC 1/6 (2.0) COS 1/6 (2.0) PRO 2/6 (2.0) CPRO 3/6 (2.0) | 0.314 |
| d 2         | NC 2/6 (2.0) PC 0/6 (0.0) COS 1/6 (2.0) PRO 2/6 (2.0) CPRO 3/6 (2.0) | 0.356 |
| d 3         | NC 3/6 (2.0) PC 0/6 (0.0) COS 1/6 (2.0) PRO 2/6 (2.0) CPRO 1/6 (2.0) | 0.144 |

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 × 10⁶ 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; ETEC K88⁺ strain. 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., 2008), 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.
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