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Weaned pig responses to *Escherichia coli* K88 oral challenge when receiving a lysozyme supplement[1,2]

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ABSTRACT: Lysozyme is a low-molecular-weight protein with antimicrobial properties. An experiment was conducted to investigate the response of piglets receiving a water-soluble lysozyme supplement [Entegard (EG), Neova Technologies Inc., Abbotsford, British Columbia, Canada; 4,000 lysozyme units/mg] after oral challenge with enterotoxigenic *Escherichia coli* (ETEC). A total of 36 individually housed weanling pigs were randomly allotted to 1 of the 4 treatments, with 9 replicates per treatment. Treatments were a control (CONT, no additive), antibiotic (AB; 2.5 g/kg of feed of antibiotic with chlortetracycline, sulfamethazine, and penicillin), and EG delivered in the drinking water at concentrations of 0.1% (EG1) and 0.2% (EG2). All pigs received a basal diet similar in composition and nutrients, except for pigs receiving the AB diet, which had an added antibiotic. Pigs were acclimated to treatments for a 7-d period to monitor growth performance. On d 8, blood samples were collected from each pig to obtain serum, and each pig was gavaged with 6 mL (2×10^9 cfu/mL) of ETEC solution. Pigs were monitored for another 7 d to assess incidences of diarrhea and growth performance, and then all pigs were killed to obtain intestinal tissue and digesta samples. Treatments did not influence growth performance throughout the study. Greater ETEC counts were observed in the ileal mucosal scrapings ($P = 0.001$) and colonic digesta ($P = 0.025$) of pigs in the CONT group compared with pigs in the AB and EG1 groups. Pigs receiving AB and EG1 had greater ($P < 0.05$) small intestinal weights and ileal villus heights than pigs receiving CONT; however, the ileal villus height-to-crypt depth ratio was greater in pigs fed the AB diet (1.69) compared with those fed the CONT diet (1.34), whereas pigs receiving EG1 were intermediate. Pigs in the EG1 group showed greater ($P < 0.001$) serum tumor necrosis factor α and IL-6 concentrations before ETEC challenge; however, at 7 d postchallenge, pigs receiving EG2 showed the least ($P < 0.05$) circulating tumor necrosis factor α and IL-6 concentrations. Overall, better intestinal growth and development, as well as decreased ETEC counts on the intestinal mucosa and serum proinflammatory cytokines, suggest that EG can maintain gut health and function in piglets commensurate with antibiotics. However, it is noteworthy that at the largest dose tested, EG seemed to have a dramatic effect on proinflammatory cytokines but had a minimal or no effect on the other response criteria.

Key words: *Escherichia coli* K88$^+$, gut health, lysozyme, piglet, postweaning diarrhea

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INTRODUCTION

Pig starter diets containing in-feed antibiotics have been used to control incidences of diarrhea and as growth promoters in young pigs (Pluske et al., 2002; de Lange et al., 2010). However, because of increased public pressure to discontinue the use of in-feed antibiotics in livestock diets, the need to identify effective alternative therapies has been the focus of several recent studies (Owusu-Asiedu et al., 2003; Bhandari et al., 2008, Kiarie et al., 2009, 2011).

Sow milk contains many factors with nonspecific antimicrobial and immune-stimulating activities, including lysozyme, a low-molecular-weight protein with bactericidal activity (Losso et al., 2000; Salmon et al., 2009). In a study using the piglet as a model for humans, Brundige et al. (2008) showed that feeding goat milk with a greater concentration of human lysozyme

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may confer gut health benefits and prevent *Escherichia coli* infection. Similarly, hen egg lysozyme was shown to modulate the immune response, maintain gut barrier function, and reduce the negative effects of dextran sodium sulfate-induced colitis in weaned pigs (Lee et al., 2009). Enterotoxigenic *E. coli* (**ETEC**), the main causative agent for diarrhea in weaned pigs, attach and efface the intestinal mucosa, thus leading to malabsorption of large molecules as a result of a compromised barrier function. In a preliminary in vitro study, a water-soluble lysozyme supplement (4,000 lysozyme units/mg) was shown to be effective against ETEC (G. Zhang, unpublished data), but this observation has not been confirmed in vivo.

Thus, the present study tested the hypothesis that a water-soluble lysozyme supplement would prevent infection and improve performance when piglets were challenged with ETEC, and the objective of the current study was to determine the response to an ETEC challenge when piglets consuming nonmedicated diets were provided with drinking water containing a water-soluble lysozyme supplement at 2 concentrations. A commercial in-feed antibiotic was tested for comparison.

MATERIALS AND METHODS

The animal protocol for this research was reviewed and approved by the University of Manitoba Animal Care Committee and followed the principles established by the Canadian Council on Animal Care (2009).

Animals and Housing

A total of 36 [(Yorkshire × Landrace) female × Duroc male; Genesus, Oakville, Manitoba, Canada] piglets weaned at 17 ± 1 d were obtained from the Glenlea swine research unit at the University of Manitoba. The initial BW of the piglets was 4.86 ± 0.41 kg. Pigs were individually housed in pens within a room in the T. K. Cheung Centre for Animal Science Research and were blocked on the basis of BW (Kim and Lindemann, 2007). All pens were equipped with a feeder, a nipple-type drinker (with some modification), and plastic-covered expanded metal floors. Room temperature was maintained at 29 ± 1°C throughout the study.

Experimental Treatments and Allocation

Additives were provided either in water [Entegard (**EG**), a water-soluble lysozyme supplement, 4,000 lysozyme units/mg; Neova Technologies Inc., Abbotsford, British Columbia, Canada) or in feed [antibiotic (**AB**); Aureo S-P 250, Alpharma Animal Health, Bridgewater, NJ]. All piglets were fed a basal diet (Table 1) with similar ingredients and nutrient composition, except for piglets receiving the AB treatment, in which the antibiotic replaced a small amount of soybean meal. The basal diet was formulated to meet or exceed NRC (1998) nutrient specifications for pigs weighing 5 to 10 kg. There were 4 experimental treatments: the control (**CONT**, no in-feed or in-water additive), AB (2.5 g of Aureo S-P 250/kg of feed, Alpharma Animal Health), and 0.1% EG (**EG1**) and 0.2% EG (**EG2**) in water. The appropriate dose of EG was dissolved in water and placed in a jug (approximately 10 L) connected to the water delivery system. A steady supply of EG-supplemented water was maintained throughout the day by occasionally filling the jug. The jugs were emptied of the remaining EG water every morning, rinsed, and refilled with fresh preparations. Pigs in the groups that did not receive in-water treatments had a similar water delivery system setting to ensure reproducibility of the experimental conditions. The 4 experimental treatments were allotted in a completely randomized design to give 9 pens per treatment (Kim and Lindemann, 2007). Pigs had unlimited access to feed and water throughout the study.

Performance Monitoring, ETEC Oral Challenge, Blood Samples, and Diarrhea Assessment

Pigs were initially exposed to treatments for 7 d, during which feed intake and BW were monitored to evaluate preinfection performance. On d 8, a 10-mL blood sample from each pig was collected into a nonheparinized Vacutainer tube (Becton Dickinson, Rutherford,

Table 1. Composition and nutrient analysis of experimental diets (as-fed basis)[1]

Item	CONT	AB
Ingredient, %		
Corn	24.00	24.00
Soybean meal	22.32	22.07
Wheat	21.00	21.00
Canola oil	5.00	5.00
Dried whey	20.00	20.00
Fish meal	5.00	5.00
Limestone	0.65	0.65
Monocalcium phosphate	0.27	0.27
Salt	0.25	0.25
Vitamin-trace mineral premix[2]	1.00	1.00
L-Lys·HCl	0.30	0.30
L-Thr	0.05	0.05
DL-Met	0.16	0.16
Antibiotic	—	0.25
Nutrient analysis		
CP, %	20.8	20.6

[1]CONT = control, no additive; AB = antibiotic, Aureo S-P 250 (Alpharma Animal Health, Bridgewater, NJ) at 2.5 mg/kg of control feed.

[2]Provided the following per kilogram of complete diet: 9,000 IU of vitamin A; 1,500 IU of vitamin D_3; 18 mg of vitamin E; 1.5 mg of vitamin K; 250 mg of choline; 30 mg of niacin; 27.5 mg of calcium pantothenate; 9.4 mg of riboflavin; 2 mg of pyridoxine; 25 μg of cyanocobalamin; 80 μg of biotin; 0.5 mg of folic acid; 18 mg of copper from copper sulfate, 110 mg of zinc from zinc oxide, 0.2 mg of iodine from calcium iodide, 110 mg of iron from ferrous sulfate, 50 mg of manganese from manganese dioxide, and 0.3 mg of selenium from sodium selenite.

NJ) and stored on ice for 20 min before being centrifuged at 3,000 × *g* for 10 min at 4°C to obtain serum for baseline (prechallenge) analysis of cytokines. Serum samples were stored at −80°C until required for analysis. Subsequently, all pigs were orally challenged with ETEC (*E. coli* K88$^+$). The ETEC strain was originally obtained from the university laboratory (C. Gyles, University of Guelph, Guelph, Ontario, Canada). To evaluate the proliferation of ETEC and to differentiate the inoculum from the indigenous strains, the pure ETEC strain was made resistant to ciprofloxacin by exposing it to increasing doses of ciprofloxacin in Müller-Hinton broth (Becton, Dickinson and Company, Sparks, MD) as described by Opapeju et al. (2009). Before being used to challenge pigs, the ciprofloxacin-resistant ETEC was confirmed to be positive for K88 fimbrial antigen, heat-labile enterotoxin, and heat-stable enterotoxin genes by PCR genotyping using published primers (Kotlowski et al., 2007; Setia et al., 2009). Each pig was orally challenged with 6 mL (2×10^9 cfu/mL) of the freshly grown ETEC inoculants via a polyethylene tube attached to a syringe placed in the back of the oral cavity. The bacteria-rich PBS solution was slowly dribbled into the throat of the pig so that the swallowing reflex was triggered and the chance of passage of the inoculant into the lungs was minimized.

To monitor postchallenge growth performance, feeders were emptied and BW was recorded 2 h before ETEC challenge. Immediately after ETEC challenge, feeders were refilled with the respective diets. At 7 d postchallenge, all pigs and the uneaten feed were weighed to evaluate postchallenge performance. The occurrence and severity of postweaning diarrhea were monitored and assessed on a pen basis by using a fecal consistency scoring system (0 = normal; 1 = soft feces; 2 = mild diarrhea; 3 = severe diarrhea; Marquardt et al., 1999) at 6, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h postchallenge by 2 trained personnel with no prior knowledge of the dietary treatment allotment.

Tissue and Digesta Samples Collection

On d 7 postchallenge, all pigs were killed as described previously by Kiarie et al. (2007), and the abdominal cavity was opened from the sternum to pubis to expose the whole gastrointestinal tract (**GIT**). The spleen and liver were removed, blotted dry with paper towels, and weighed. The GIT was separated into 4 segments (stomach, small intestine, cecum, and colon) using clamps to minimize digesta movement. The small intestine was stripped free of its mesentery and further divided into 2 sections. The ileum included the intestine from the ileal-cecal junction to 80 cm cranial to this junction, and the remaining portion was designated as the duodenum and jejunum. The colon was further sectioned into the proximal colon (from the cecal-colonic junction to the apex of the spiral colon) and distal colon (from the apex of the spiral colon to the rectum).

Approximately 5 cm of ileal tissue was cleaved, placed in a sterile container, and transferred to the laboratory (within 30 min of collection) for enumeration of bacteria attached to the mucosal scrapings. Another section of ileum was removed and fixed by immersion in carnoy solution (ethanol, chloroform, and acetic acid; 6:3:1; 4°C) for histomorphology analysis (Puchtler et al., 1968).

Thereafter, all sections were emptied of their digesta and weighed. Digesta contents of the distal colon were emptied into 2 separate sterile sample bags, and those of the ileum were emptied into 1 sterile sample bag. For the distal colon samples, 1 bag was placed on ice and transferred to the laboratory immediately for microbial count. The pH was determined in the ileum digesta and the colon digesta (second bag) by using an electronic pH meter (Accumet Basic, Fisher Scientific, Fairlawn, NJ). Thereafter, digesta from the distal colon was stored at −20°C until required for analysis of organic acids.

Laboratory Analyses

Intestinal Histomorphology. After 3 h in carnoy solution, samples were transferred to vials containing 80% ethanol and sent for processing to a commercial laboratory (Cancer Care Manitoba, Winnipeg, Manitoba, Canada). Villus height (**VH**), from the tip of the villi to the villus-crypt junction, and the crypt depth (**CD**), from the villus-crypt junction to the base of the crypt, were measured at 10× magnification (Axiostar Plus microscope, Carl Zeiss, Oberkochen, Germany) equipped with a camera (Canon Canada Inc., Mississauga, Ontario, Canada) and image software (National Institutes of Health, Bethesda, MD) in at least 15 well-oriented villus and crypt columns. The VH:CD ratio (**VCR**) was calculated.

Organic Acid Concentration of Colonic Digesta. Organic acids (acetic, propionic, butyric, isobutyric, valeric, isovaleric, and lactate) were assayed using gas chromatography according to Erwin et al. (1961). Briefly, an aliquot of 2.5 mL of digesta fluid was mixed with 0.5 mL of 25% metaphosphoric acid in a centrifuge tube and the mixture frozen overnight. Thawed samples were mixed with 200 μL of 25% NaOH and vortexed, followed by addition of 320 μL of 0.3 *M* oxalic acid. The samples were then centrifuged at 3,000 × *g* for 20 min at room temperature and the supernatant (2 mL) was transferred to GLC vials. The organic acids were determined using a glass column packed with 80/120 Carbopack B-DA/4% Carbowax 20M (Supelco, Bellefonte, PA) in a gas chromatograph (model 3400, Varian Inc., Palo Alto, CA).

Gut Microbial Analysis. Ileal segments were aseptically opened longitudinally and the mucosa was scraped from the luminal surface by using a sterile glass microscope slide. One gram of ileal mucosal scrapings was transferred in 15-mL tubes containing beads and 9

Table 2. Effect of antibiotic and lysozyme on growth performance and fecal consistency scores of piglets challenged with *Escherichia coli* K88^{+1}

	Treatment[2]					
			Entegard supplement[3]			
Item	CONT	AB	EG1	EG2	SEM	*P*-value
Initial BW, kg	4.92	4.90	4.90	4.72	0.14	0.76
ADG, g/d						
Prechallenge	75	138	86	86	20	0.14
Postchallenge	117	209	183	135	32	0.19
ADFI, g/d						
Prechallenge	115	153	117	156	17	0.22
Postchallenge	186	278	237	218	33	0.24
G:F, g/g						
Prechallenge	0.64	0.83	0.77	0.54	0.15	0.56
Postchallenge	0.66	0.79	0.74	0.67	0.13	0.86
Fecal consistency score,[4] time postchallenge						
6 h	1.07	0.97	0.40	0.95	0.27	0.32
24 h	1.30	1.04	0.88	1.10	0.27	0.74
36 h	1.73	1.18	1.02	1.45	0.30	0.37
48 h	1.73	1.21	0.93	1.36	0.28	0.26
60 h	1.73	1.40	0.90	1.46	0.30	0.27
72 h	1.71	1.41	0.87	1.26	0.29	0.24
84 h	1.71	1.43	0.87	1.26	0.29	0.24
96 h	1.44	1.19	0.86	1.16	0.30	0.62
108 h	1.26	0.70	0.86	0.98	0.25	0.46
120 h	0.94	0.48	0.60	0.74	0.26	0.66
Average	1.49	1.08	0.82	1.16	0.25	0.31

[1]Piglets exposed to treatments at weaning and challenged with *E. coli* K88^{+} on d 8. Prechallenge = weaning to d 7; postchallenge = d 8 to 14.

[2]CONT = control, no dietary or drinking water additive; AB = antibiotic, Aureo S-P 250 (Alpharma Animal Health, Bridgewater, NJ) at 2.5 mg/kg of control feed and no drinking water additive; EG1 = control diet + 0.1% Entegard supplement (Neova Technologies Inc., Abbotsford, British Columbia, Canada) in drinking water; EG2 = control diet + 0.2% Entegard supplement (Neova Technologies Inc.) in drinking water. n = 9.

[3]Supplement: 4,000 lysozyme units/mg.

[4]Scores: 0 = normal; 1 = soft feces; 2 = mild diarrhea; 3 = severe diarrhea (Marquardt et al., 1999).

mL of peptone water (Fisher Scientific, Fairlawn, NJ) and vortexed. One gram of digesta from the distal colon was added to 9 mL of sterile 0.1% peptone water, vortexed for 60 s, and then serially diluted 10-fold in sterile peptone water. Total coliforms and ETEC in the serially diluted samples were quantified using eosin methylene blue agar (Becton Dickinson & Co., Franklin Lakes, NJ) without and with ciprofloxacin (0.5 μg/mL), respectively. The plates were incubated aerobically at 37°C for 24 to 48 h.

Serum Cytokine Concentration. Serum concentrations of IL-6 and tumor necrosis factor α (**TNF-α**) were determined using commercially available kits (Quantikine Kits, R&D Systems Inc., Minneapolis, MN). All samples were analyzed in duplicate within a single assay. Detection limits were 10 and 23 pg/mL, with intraassay CV of 5.3 and 4% for IL-6 and TNF-α, respectively.

Statistical Analysis

Data for bacterial enumeration were logarithmically transformed, and data for GIT and organ weights were expressed as a proportion of BW before statistical analysis. Data were analyzed as a completely randomized design using PROC MIXED procedures (SAS Inst. Inc., Cary, NC). When a significant *F*-value ($P < 0.05$) for treatment means was observed by ANOVA, treatments were compared using Tukey's test.

RESULTS

Piglet Performance and Fecal Scores

No differences were observed among treatments in any of the performance indicators before and after challenge (Table 2). Similarly, fecal consistency scores did not differ among treatments at any time period.

Intestinal Coliforms, ETEC Counts, Digesta pH, and Organic Acids

Counts of coliforms attached to the ileal mucosa were less ($P = 0.030$) for pigs in the AB and EG1 treatments compared with those in the CONT treatment, and the count for the EG2 treatment was intermediate (Table 3). The counts for ETEC were less ($P = 0.001$) in the ileal mucosal scrapings of piglets receiving the AB and EG1 treatments compared with piglets in the CONT treatment. In colonic digesta, counts for coliforms did

Table 3. Effect of antibiotic and lysozyme on gastrointestinal microbial activity in piglets challenged with *Escherichia coli* K88^{+}[1]

	Treatment[2]					
			Entegard supplement[3]			
Item	CONT	AB	EG1	EG2	SEM	*P*-value
Total coliform count,[4] $\log_{10}$ cfu/g						
Ileal mucosal scrapings	5.60[a]	4.25[b]	4.33[b]	4.74[ab]	0.32	0.03
Colon digesta	5.52	4.58	5.19	5.48	0.55	0.59
E. coli K88^{+} count,[5] $\log_{10}$ cfu/g						
Ileal mucosal scrapings	4.75[a]	2.68[b]	2.47[b]	3.83[a]	0.40	<0.01
Colon digesta	4.42[a]	3.00[b]	2.90[b]	3.39[ab]	0.36	0.03
Digesta pH						
Ileum	6.75	6.52	7.07	6.83	0.17	0.14
Colon	6.58	6.26	6.55	6.27	0.14	0.22
Colon digesta organic acids, mmol/L						
Acetic	27.4	28.8	25.7	27.5	4.7	0.97
Propionic	13.5	17.3	14.4	17.7	1.6	0.18
BCVFA[6]	10.7	14.0	9.1	13.9	2.2	0.30
Lactic	0.19	0.20	0.10	0.45	0.17	0.50
Total[7]	51.7	60.3	49.3	59.4	6.7	0.56

[a,b]Within a row, values with different superscripts are different, $P < 0.05$.

[1]Piglets exposed to treatments throughout the entire experimental period, challenged with *E. coli* K88 on d 8, and killed on d 14 for organ and sample collection.

[2]CONT = control, no dietary or drinking water additive; AB = antibiotic, Aureo S-P 250 (Alpharma Animal Health, Bridgewater, NJ) at 2.5 mg/kg of control feed and no drinking water additive; EG1 = control diet + 0.1% Entegard supplement (Neova Technologies Inc., Abbotsford, British Columbia, Canada) in drinking water; EG2 = control diet + 0.2% Entegard supplement (Neova Technologies Inc.) in drinking water. n = 9.

[3]Supplement: 4,000 lysozyme units/mg.

[4]Plated on eosin methylene blue agar.

[5]Plated on eosin methylene blue agar containing 0.5 μg of ciprofloxacin/mL (Opapeju et al., 2009).

[6]BCVFA = branched-chain VFA, sum of isobutyrate, isovalerate, butyrate, and valerate.

[7]Sum of acetic, propionic, BCVFA, and lactic acids.

not differ among treatments, but piglets from the AB and EG1 treatments had smaller ($P = 0.025$) ETEC counts compared with those in the CONT group; values for the piglets receiving EG2 were intermediate.

No differences ($P > 0.10$) among treatments were observed in digesta pH in the ileum and colon. No differences were observed in VFA and lactic acid concentrations in the colon digesta, and only minimal changes were observed in the fermentation activities among treatments.

Visceral Organ Weights and Intestinal Morphology

Piglets in the AB and EG1 treatments had greater empty small intestinal weights than did those in the CONT treatment, and those in the EG2 treatment were intermediate (Table 4). No differences were observed among treatments for any other organ weighed.

Piglets assigned to the AB and EG1 treatments had longer ($P < 0.001$) villi compared with those in the CONT and EG2 treatments, but no differences were observed among treatments for CD. Piglets in the AB treatment had greater VCR than those in the CONT treatment. The VCR values for piglets assigned to the EG treatments were intermediate to those for piglets in the CONT and AB treatments.

Immunological Responses

Serum concentrations of TNF-α and IL-6 before and after ETEC challenge are shown in Table 5. Before ETEC challenge, pigs in the EG1 group showed greater ($P < 0.001$) TNF-α and IL-6 concentrations than those in the CONT, AB, and EG2 treatments. However, at 7 d postchallenge, pigs receiving EG2 showed ($P < 0.05$) the least concentrations of circulating TNF-α and IL-6 compared with pigs receiving the CONT and AB diets.

DISCUSSION

The goal of this experiment was to determine whether adding a lysozyme supplement to the drinking water of weaned pigs would protect against ETEC infection when the pigs consumed a starter diet with no supplemental antibiotics. Piglet growth performance was unaffected by dietary treatments. The lack of growth response attributable to the supplemental antibiotic was surprising, given the fact that we recently reported better performance in piglets fed the antibiotic after an ETEC challenge (Kiarie et al., 2011). However, previous studies with transgenic goat milk containing human lysozyme reported no improvements in piglet growth compared with milk without lysozyme (Maga et al., 2006; Brundige et al., 2008).

Table 4. Effect of antibiotic and lysozyme on visceral organ weights and ileal morphology in piglets challenged with *Escherichia coli* K88^{+1}

	Treatment[2]					
			Entegard supplement[3]			
Item	CONT	AB	EG1	EG2	SEM	*P*-value
Organ weight, g/kg of BW						
Stomach	9.16	8.93	8.81	8.33		
Small intestine	50.5^{b}	57.2^{a}	57.5^{a}	55.0^{ab}	1.784	0.05
Large intestine	22.0	20.8	20.9	24.3	1.011	0.07
GIT[4]	62.6	66.1	66.3	63.3	2.345	0.59
Liver	28.5	34.4	29.4	29.0	2.564	0.35
Spleen	2.83	2.70	2.70	2.83	0.238	0.26
Ileal morphology						
Villi height, μm	363^{b}	458^{a}	428^{a}	362^{b}	10.1	<0.01
Crypt depth, μm	276	280	285	269	18.1	0.93
Villi height:crypt depth ratio	1.34^{b}	1.69^{a}	1.56^{ab}	1.38^{ab}	0.10	0.05

^{a,b}Within a row, values with different superscripts are different, $P < 0.05$.

[1]Piglets exposed to treatments throughout the entire experimental period, challenged with *E. coli* K88^{+} on d 8, and killed on d 14 for organ and sample collection.

[2]CONT = control, no dietary or drinking water additive; AB = antibiotic, Aureo S-P 250 (Alpharma Animal Health, Bridgewater, NJ) at 2.5 mg/kg of control feed and no drinking water additive; EG1 = control diet + 0.1% Entegard supplement (Neova Technologies Inc., Abbotsford, British Columbia, Canada) in drinking water; EG2 = control diet + 0.2% Entegard supplement (Neova Technologies Inc.) in drinking water. n = 9.

[3]Supplement: 4,000 lysozyme units/mg.

[4]Gastrointestinal tract, summation of stomach, small intestine, and large intestine.

The fecal consistency scores observed in the present study are typical of the challenge model used and do not indicate that a major diarrheal episode was achieved (Bhandari et al., 2008; Kiarie et al., 2011). Because of animal welfare considerations and strict end points to be observed, our aim was to make the pigs moderately ill to allow evaluation of the potential protective effects of various therapies. Furthermore, the presence or absence of K88 receptor (not determined in the current study) in the pig intestine has been associated with variability in ETEC colonization in the gut (Geenen et al., 2007).

Lysozyme is a 14.6-kDa single peptide that can result in cell lysis by enzymatically cleaving the β(1–4) glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall (Proctor et al., 1988). It has been shown to be effective against various pathogens, including *E. coli* (Ellison and Giehl, 1991; Salmon et al., 2009). The results of the present study support these observations and are consistent with our preliminary in vitro observations (G. Zhang, unpublished data). Because of its antimicrobial properties, we had hypothesized that lysozyme delivered in drinking water would

Table 5. Effect of antibiotic and lysozyme on serum concentrations of tumor necrosis factor α (TNF-α) and IL-6 of piglets challenged with *Escherichia coli* K88^{+1}

	Treatment[2]					
			Entegard supplement[3]			
Item	CONT	AB	EG1	EG2	SEM	*P*-value
TNF-α, pg/mL						
Prechallenge	111.4^{b}	62.9^{c}	193.1^{a}	99.8^{b}	8.7	<0.01
Postchallenge	162.1^{a}	150.0^{a}	132.4^{a}	89.9^{b}	9.4	<0.01
IL-6, pg/mL						
Prechallenge	48.0^{b}	25.8^{b}	159.7^{a}	41.9^{b}	7.3	<0.01
Postchallenge	85.8^{a}	81.2^{a}	60.7^{ab}	48.6^{b}	7.0	<0.01

$^{a–c}$Within a row, values with different superscripts are different, $P < 0.05$.

[1]Piglets exposed to treatments at weaning and challenged with *E. coli* K88^{+} on d 8. Prechallenge = morning of d 8 before challenge; postchallenge = morning of d 14.

[2]CONT = control, no dietary or drinking water additive; AB = antibiotic, Aureo S-P 250 (Alpharma Animal Health, Bridgewater, NJ) at 2.5 mg/kg of control feed and no drinking water additive; EG1 = control diet + 0.1% Entegard supplement (Neova Technologies Inc., Abbotsford, British Columbia, Canada) in drinking water; EG2 = control diet + 0.2% Entegard supplement (Neova Technologies Inc.) in drinking water. n = 9.

[3]Supplement: 4,000 lysozyme units/mg.

prevent the intestinal proliferation of ETEC in piglets consuming an antibiotic-free diet in much the same manner as in-feed antibiotics. The results of the present study support this hypothesis in that the counts of total coliforms and ETEC adhering to ileal mucosa were less for piglets in the AB and EG1 groups compared with those in the CONT group. In our challenge model, we used an ETEC that was intentionally made resistant to a specific antibiotic to allow a distinction to be made between the total coliform and ETEC counts in a sample (Bhandari et al., 2008, 2009; Kiarie et al., 2009, 2011). For ETEC to cause intestinal infection, it must first attach to the intestinal mucosa by using its fimbria (Fairbrother et al., 2005). Our results indicated that EG at the smaller dose prevented the attachment of ETEC to the intestinal mucosa, thus minimizing its associated damage, which is consistent with the intestinal morphology results obtained in the present study. Various studies have shown that ETEC infections increase intestinal permeability, thus reducing its barrier function (Kiarie et al., 2008a,b). Because lysozyme reduced ETEC associated with the ileal mucosa, it is possible that this was accompanied by reduced gut permeability and an enhanced gut barrier function, as observed by Lee et al. (2009). Although coliform counts in the colon digesta did not differ among treatments, counts for ETEC followed the same trend as for ileal mucosal-adherent ETEC. Overall, the results of the present study are consistent with those of Brundige et al. (2008), who reported that piglets consuming transgenic goat milk containing human lysozyme had smaller coliform and *E. coli* counts compared with those fed control milk after an enteropathogenic *E. coli* challenge. Similarly, Maga et al. (2006), who fed 19-d-old weaned pigs lysozyme-containing milk from transgenic goats for 16 d, found smaller coliform and *E. coli* counts in the duodenal contents. The presence of coliforms in the small intestine of piglets is usually a result of infection because healthy piglets generally have a greater percentage of gram-positive bacteria than gram-negative bacteria in the small intestine (Pluske et al., 2002; Bhandari et al., 2008). Decreases in coliform counts are important because certain toxins produced by coliforms produce intestinal hyperactivity, secretion, and diarrhea (Giannella, 1983).

Intestinal growth and development are critical for the optimal performance of young swine. Furthermore, reductions in VH have been associated with poor growth performance and increased incidences of scouring in pigs challenged with ETEC (Owusu-Asiedu et al., 2003). The small intestinal weights in the present study show that supplementing EG at 0.1% in drinking water maintained the growth of this organ to the same extent as did the AB treatment. This observation is consistent with the intestinal morphology results obtained in the present study, which showed longer VH in piglets receiving AB and EG1 than in piglets receiving the CONT diet. Longer VH are often used as an indicator of an increased absorptive capacity of the small intestine and a healthy gut (Pluske et al., 1997; Nyachoti et al., 2006; Kiarie et al., 2009). Humphrey et al. (2002) reported in a broiler study that dietary supplementation with an increased quantity of human lysozyme increased the VH in the duodenum, which is consistent with the ileal results of piglets receiving EG1 in the present study. The renewal of the intestinal epithelium is a consequence of a dynamic equilibrium between the production of enterocytes in the crypt and desquamation in the villus (Willing and Van Kessel, 2007). Thus, the VCR is a useful criterion for assessing intestinal health and function (Pluske et al., 1997). Studies have indicated that lysozyme contributes to the establishment of bifidobacterium- and lactobacillus-rich gut biota in breast-fed infants that are protective against gut illness and may aid in the maturation of the intestinal tract (Schiffrin and Blum 2002; Newburg and Walker, 2007). Furthermore, a feature of ETEC infection is effacing of the intestinal mucosa, which often leads to shorter villi and deeper crypts (Fairbrother et al., 2005). These might explain why piglets consuming water containing EG had VCR similar to those of piglets fed the AB diet, indicating that providing EG in the drinking water was able to prevent ETEC from colonizing and damaging the intestinal mucosa in piglets consuming the CONT diet.

Lysozyme is an important nonspecific immmunomodulating factor in mammalian milk (Salmon et al., 2009), whereas ETEC colonization is a potent immune stimulant (Kiarie et al., 2009). Hence, serum concentrations of TNF-α and IL-6 were determined to assess whether EG could mitigate the immunological responses associated with ETEC infection in piglets. These proinflammatory cytokines play a critical role in normal host resistance to infection, serving as immmunomodulators and as mediators of inflammatory responses. They are primarily produced by activated macrophages through numerous signals, such as mitogenic or antigenic stimulation, lipopolysaccharide, calcium ionophores, cytokines, and viruses (Baumann and Gauldie, 1994). Products of bacterial cells after hydrolysis by lysozyme include muramyl dipeptide, a potent adjuvant capable of enhancing IgA secretion, macrophage activation, and rapid clearance of bacterial pathogens in vivo (Kawano et al., 1981). Perhaps activation of macrophages might have mediated greater TNF-α and IL6 production in the EG1 treatment before challenge. The downregulation of TNF-α and IL-6 in the presence of EG at the greater dose indicated that ETEC-induced inflammation was minimized compared with when EG was absent. This observation is consistent with the results of the study by Lee et al. (2009) showing that hen egg lysozyme reduced the expression of proinflammatory cytokines in the colonic tissue, thus minimizing tissue damage and consequently improving barrier function and reducing gut permeability. Generally, these data are consistent with the intestinal *E. coli*

counts and would indicate that the lysozyme product evaluated was able to minimize ETEC infection in piglets.

It is noteworthy that EG at the largest dose tested seemed to have a dramatic effect on proinflammatory cytokines but a minimal or no effect on the other response criteria evaluated. It is difficult to explain these observations, indicating that further research is needed to understand the dose response of lysozyme in piglets.

The current study contributes to an understanding of the mechanisms through which lysozyme may benefit piglets during ETEC challenge. The results of the current study show that providing weaned pigs with drinking water containing a smaller dose of EG was effective in minimizing the proliferation of pathogenic ETEC in the ileum and in protecting the intestinal mucosa against ETEC damage, as indicated by the data on intestinal morphology and ETEC count associated with the ileal mucosa. The EG at a larger dose also modulated the immunological response in piglets challenged with ETEC and seemed to prevent inflammation.

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