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Bacteriophage cocktail and multi-strain probiotics in the feed for weanling pigs: effects on intestine morphology and targeted intestinal coliforms and *Clostridium*

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Two experiments were conducted to investigate the effects of dietary supplementation of bacteriophage cocktail, probiotics and a combination of these two supplements on performance and gut health of weanling pigs. In Experiment 1, 150 weaned piglets were randomly allotted to three treatments on the basis of BW. The dietary treatments included a basal diet supplemented with 0 (control), 1.0 and 1.5 g/kg bacteriophage cocktail. Pigs fed 1.0 and 1.5 g/kg bacteriophage product had greater ($P < 0.05$*) average daily gain (ADG), apparent total tract digestibility of dry matter from day 22 to 35, ileal* Lactobacillus *spp., villus height (duodenum and jejunum), and fewer coliforms (ileum) and* Clostridium *spp. (ileum). In Experiment 2, 200 weaned piglets were randomly allotted to four treatments. Dietary treatments included basal diet, basal diet supplemented with 3.0 g/kg fermented probiotic product (P), 1.0 g/kg bacteriophage cocktail (B) and combination of 1.0 g/kg bacteriophage cocktail and 3.0 g/kg fermented probiotic product. Pigs fed bacteriophage cocktail diets had greater (*$P < 0.05$*) overall ADG, gain to feed ratio (G : F), fecal score from day 8 to day 21, and pigs fed bacteriophage cocktail diets had fewer coliforms (ileum)* Clostridium *spp. (ileum and cecum). Probiotics significantly increased G : F, colonization of* Lactobacillus *spp. in ileum. At day 35, bacteriophage treatment group showed greater (*$P < 0.05$*) villus height of the duodenum, but a deeper crypt in duodenum. The present results indicate that the bacteriophage cocktail had a potential to enhance the performance and gut health of weanling pigs, however their combination with probiotics did not show an interaction.*

Keywords: bacteriophage, microflora, intestinal morphology, probiotics, weanling pigs

Implications

In this study, dietary bacteriophage was aimed to act against coliforms and *Clostridium* spp. to avoid their proliferation in unstable intestinal microbiota after weaning, when their feed form changes from milk to mash. To our knowledge, no previous work has been surveyed the effect of bacteriophage cocktail against four different pathogens in the weaning period, furthermore no information is available regarding the effect of bacteriophages on intestinal morphology of weanling piglets. This approach has provided new insights into the use of a bacteriophage cocktail to improve the gut health of piglets.

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Introduction

At weaning, piglets are exposed to nutritional, environmental, physiological and social stressors that can lead to a depressed feed intake, a high incidence of diarrhea, poorer growth performance and increased mortality of piglets (Frydendahl, 2002; Halas *et al.*, 2007). To counteract these effects, antibiotic growth promoters are commonly added to weanling pig diets to maintain gut health and to improve growth performance. However, persistent use of antibiotics in animal feed has resulted in problems like emergence of drug resistant bacteria, imbalance of normal intestinal microflora and antibiotic-residue in animal products (Schwarz *et al.*, 2001), which has led to the total ban or restriction on the use of antibiotic growth promoters in many countries including the Republic of Korea (Pettigrew, 2006; GAIN, 2011). Therefore, the search continues for non-antibacterial growth promoters that are active *in vivo*, are fast acting, possess a broad spectrum in activity, do not

induce bacterial resistance and subsequently promote growth performance of pigs. A number of research findings on the use of alternatives like probiotics, oligosaccharides, organic acids and antimicrobial peptides to replace antibiotics in feed have been documented with varying success (Kenny *et al*., 2011; Choi *et al*., 2011a; Yoon *et al*., 2012). In this context, bacteriophages are believed to be an ideal candidate, due to their natural antibacterial properties (Jamalludeen *et al*., 2009; Yan *et al*., 2012).

Bacteriophages are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery (McGrath *et al*., 2004). Most of the previous studies on bacteriophages evaluated their therapeutic effects on disease challenged pigs (Barrow, 2001; Jamalludeen *et al*., 2009; Wall *et al*., 2010) only against one pathogen bacteria, but in the current experiment the bacteriophage cocktail included ten different bacteriophages against *Salmonella*, Coliforms, *Streptococcus* and *Clostridium*. The application of a bacteriophage cocktail can be a better way to prevent the proliferation of pathogens in gut microflora to avoid incidence of diarrhea and other related diseases. Recently, it has been reported that supplementation with bacteriophages resulted in improved growth performance and gut health of growing pigs (Yan *et al*., 2012; Kim *et al*. 2014b). Previous studies in the author's laboratory reported that the multimicrobial probiotic products had potential to improve the performance and gut health, and could be used as an alternative to antibiotic growth promoters in pigs and broilers (Choi *et al*., 2011b; Kim *et al*., 2012). Therefore, the present study was designed to investigate the effects of dietary supplementation with bacteriophage and also its combination with fermented probiotic product on growth performance, nutrient digestibility, intestinal and fecal microbiota and intestinal morphology of weanling pigs.

Material and methods

Bacteriophages

The bacteriophage product used in the present study was obtained from a commercial feed company (CTC Bio Inc., Seoul, Republic of Korea) by mixing of excipients with lyophilized bacteriophage cocktail infecting *Salmonella* (*S. typhimurium, S. enteritidis, S. cholerasuis and S. derby*), *Staphylococcus aureus*, *Escherichia coli* (k88, k99 and f41) and *Clostridium perfringens* types A and C. These bacteriophages are isolated from water, soil and farm waste samples and their antibacterial activities were confirmed by a conventional plaque assay. The titer of each bacteriophage in the bacteriophage cocktail was 10^9 plaque-forming units (pfu)/g bacteriophage cocktail.

Preparation of fermented probiotic product

Lactobacillus acidophilus K31 isolated from feces of weaned pigs, *Bacillus subtilis* K 42 isolated from natto (fermented soybeans), and *Saccharomyces cerevisiae* K47 isolated from koji (malted wheat) were maintained in the laboratory as stock culture. A culture broth (CB) medium containing 60.0 ml corn steep liquor, 40.0 ml molasses, 3.0 g/l yeast extract, 5.0 g/l KH_2PO_4 and 2.5 g/l K_2HPO_4 in distilled water was prepared and autoclaved before being used.

A quantity of 2 l of autoclaved CB was inoculated with 2.0 ml of culture of each microbe separately and subjected to fermentation for 48 h. *L. acidophilus* and *B. subtilis* were incubated at 37°C at pH 7.0, whereas *S. cerevisiae* was incubated at 3°C at pH 4.0. The microbes grown on CB were directly sprayed on corn-soybean meal (1 : 1) followed by drying at 40°C for 72 h.

The microbes grown on CB were used as starter and pasteurized corn : soybean meal (1 : 1) was used as the substrate for carrying out fermentation as described previously by Shim *et al*. (2010). Then the substrates (13.0 kg) were inoculated with 2.0 l of starter and fermented for 7 days at 32°C and at pH 7.0. After 7 days of fermentation, the complete fermentation batch was dried at 40°C for 72 h and mixed to obtain the fermented probiotic product. The colony counts of *L. acidophilus*, *B. subtilis* and *S. cerevisiae* in fermented probiotic product (Shim *et al*., 2010) were 4.0×10^8, 4.8×10^9 and 1.0×10^4 colony-forming units (cfu)/g, respectively.

Animals, diets and management

In Experiment 1, a total of 150 weaned piglets (Landrace × Yorkshire × Duroc; initial BW: 7.77 ± 0.250 kg; 24 ± 3 days of age) of mixed sex were randomly allotted to three treatments on the basis of BW and sex. There were five replicates pens in each treatment with 10 pigs per pen. The dietary treatments included a basal diet supplemented with 0 (control diet without any antimicrobial), 1.0 and 1.5 g/kg commercial bacteriophage product (10^9 pfu/g). Whereas, in Experiment 2, 200 weaned piglets (Landrace × Yorkshire × Duroc; initial BW; 7.76 ± 0.280 kg; 24 ± 3 days of age) of mixed sex were randomly allotted to four treatments on the basis of BW and sex. There were five replicates pens in each treatment with 10 pigs/pen. Dietary treatments included basal diet without any antimicrobial, basal diet supplemented with 3.0 g/kg fermented probiotic product (P), basal diet supplemented with 1.0 g/kg bacteriophage (B) and basal diet supplemented with combination of 1.0 g/kg bacteriophage and 3.0 g/kg fermented probiotic product. Fermented probiotic products used herein contained *L. acidophilus*, *B. subtilis* and *S. cerevisiae*. In both experiments, treatment diets were fed in a meal form in three phases (day 0 to day 7, phase I; day 8 to day 21, phase II and day 22 to day 35, phase III). Diets for phases I, II and III were formulated to contain 14.23 MJ/kg metabolizable energy and 16.0, 14.5 and 14.0 g/kg lysine, respectively (Table 1). All diets met or exceeded the nutrient requirements as suggested by NRC (1998).

The project underwent proper ethical standards and the experiments were approved by the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Republic of Korea. These experiments were conducted at the facility of Kangwon National University

Table 1 *Ingredient and chemical composition of basal diets (as-fed basis; Experiments 1 and 2)*

Items	Phase I (day 0 to day 7)	Phase II (day 8 to day 21)	Phase III (day 22 to day 35)
Ingredient (g/kg diet)			
Corn	424.5	499.5	572.3
Corn starch	80.0	–	–
SBM (dehulled)	120.0	327.4	310.0
Full fat soy	50.0	–	–
Soy oil	12.9	25.6	35.4
Whey powder	100.0	46.2	–
Lactose	20.0	20.0	–
Fish meal	50.0	50.0	50.0
Improved soybean protein	80.0	–	–
Sucrose	30.0	–	–
L-Lysine HCl (78%)	4.2	2.9	2.2
DL-Methionine (98%)	1.5	1.5	1.5
Choline chloride (50%)	1.0	1.0	1.0
Monocalcium phosphate	9.9	10.0	11.3
Limestone	9.0	8.9	9.3
Salt	2.0	2.0	2.0
Vitamin premix[1]	2.5	2.5	2.5
Mineral premix[2]	2.5	2.5	2.5
Chemical composition (calculated)[3]			
ME (MJ/kg)	14 225	14 225	14 225
CP (%)	23.0	22.0	21.0
Ca (%)	0.8	0.8	0.8
Available phosphorus	0.5	0.5	0.5
Lys (%)	1.6	1.45	1.4
Met + Cys (%)	0.8	0.8	0.7

SBM = Soybean meal; ME = metabolizable energy.
[1]Supplied per kilogram of diet: 16 000 IU vitamin A, 3000 IU vitamin D_3, 40 IU vitamin E, 5.0 mg vitamin K_3, 5.0 mg vitamin B_1, 20 mg vitamin B_2, 4 mg vitamin B_6, 0.08 mg vitamin B_{12}, 40 mg pantothenic acid, 75 mg niacin, 0.15 mg biotin, 0.65 mg folic acid.
[2]Supplied per kilogram of diet: 45 mg Fe as ferrous sulfate, 0.25 mg Co as cobalt sulfate, 50 mg Cu as copper sulfate, 15 mg Mn as manganese oxide, 25 mg Zn as zinc oxide, 0.35 mg I as potassium iodide, 0.13 mg Se as sodium selenite.
[3]Based on NRC (1998) values.

farm and the piglets were housed in partially slotted and concrete floor pens with a pen size of 1.90 × 3.0 m. All pens were equipped with a self-feeder and nipple drinker to allow *ad libitum* access to feed and water.

Experimental procedures, measurements and analyses

Individual weanling piglets weight and feed disappearance from each pen were recorded at the beginning of the experiment and at the end of every phase to calculate average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G : F). To evaluate the effects of dietary treatments on the apparent total tract digestibility (ATTD) of nutrients, 0.25% chromic oxide (an inert indigestible indicator) was added to all three phases (Phase I, 0 to 7 days; phase II, 8 to 21 days and phase III, 22 to 35 days) diets of each experiments. Pigs were fed diets mixed with chromic oxide from day 0 to day 7, day 14 to day 21 and day 28 to day 35, and fecal grab samples were collected from each pen on the last 3 days of each experiment to determine the ATTD of dry matter (DM), gross energy (GE) and CP. The fecal samples were pooled within pen and dried in a forced air oven at 60°C for 72 h, and ground in a Wiley mill (Thomas Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) using a 1 mm screen and used for chemical analysis.

The incidence of diarrhea was measured by scoring the feces as 0 (normal), 1 point (soft feces), 2 points (mild diarrhea), 3 points (severe diarrhea) in all the experiments. The overall cumulative incidence of diarrhea was measured daily at 0900 h for 5 weeks and the final diarrhea incidence was determined as the average of the scores.

To study the effects of dietary treatments on small intestinal morphology and microbiota of ileal and cecal digesta, representative piglets from each group (two per pen) reflecting the average BW of the pen were selected and sacrificed by electrocution at day 35 of each experiment. The digesta from the ileum and cecum were collected in sterile plastic bottles for microbial analysis. The samples collected for microbial analysis were immediately placed on ice until analyses were conducted. The samples of the intestinal segment from the region of duodenum, jejunum and ileum were collected after removing the content and flushing with physiological saline. The samples were then submerged in a fixative solution (0.1 M collidine buffer, pH 7.3) containing 3% glutaraldehyde, 2% paraformaldehyde and 1.5%

acrolein, and then brought to the laboratory to study the morphological changes.

Chemical and microbial analyses

Experimental diets and excreta samples were analyzed in triplicate for DM (Method 930.15) and CP (Method 990.03) using Association of Official Analytical Chemists (2007) methods. GE of diets and feces were measured by a bomb calorimeter (Model 1261, Parr Instrument Co., Moline, IL, USA), and chromium concentration was determined with an automated spectrophotometer (Jasco V-650; Jasco Corp., Tokyo, Japan) according to the procedure of Fenton and Fenton (1979).

The microbiological assay of ileum and cecum digesta was carried out by culturing in different media as suggested by Choi *et al.* (2011a).

One gram of the composite cecum or ileum sample was diluted with 9 ml of 1% peptone broth (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and then homogenized. Viable counts of bacteria in the samples were then conducted by plating serial 10-fold dilutions. For the determination of *Lactobacillus spp.* (using Man, Rogosa and Sharpe (MRS) agar + 0.200 g/l NaN_3 + 0.500 g/l L-cystine hydrochloride monohydrate), *Bifidobacterium spp.* (Man, Rogosa and Sharpe-nalidixic paromomycin neomycin lithium: MRS agar + nalidixic acid, paromomycin + neomycin sulphate + lithium chloride), *Clostridium spp.* (tryptose sulphite cycloserine agar) and coliforms (violet red bile agar) were used.

The microbiological assay of potential probiotic products was also carried out by culturing technique. *Lactobacillus* spp. was enumerated using MRS agar + 0.02% NaN_3 + 0.05% L-cystine hydrochloride monohydrate, *B. subtilis* by using plate count agar, *S. cerevisiae* by potato dextrose agar. The MRS agar (No. 288130), violet red bile agar (No. 216695), plate count agar (No. 247940), and potato dextrose agar (No. 213400) used were purchased from Difco Laboratories (Detroit, MI, USA). The bacterial concentrations were transformed (log) before statistical analysis.

Small intestinal morphology

Three cross-sections for each intestinal sample were prepared after staining with azure A and eosin using standard paraffin embedding procedures (Yoon *et al.*, 2012). A total of ten intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section. Villus height was measured from the tip of the villi to the villus crypt junction, and crypt depth was defined as the depth of the invagination between adjacent villi. All morphological measurements (villus height and crypt depth) were made in 10 μm increments by using an image processing and analysis system (Optimus version 6.5 software, Media Cybergenetics, North Reading, MA, USA).

Statistical analysis

In first experiment, statistical analysis was conducted using the one-way ANOVA procedure (SAS Institute Inc., Cary, NC, USA), and when significant differences ($P < 0.05$) were identified among treatment means, they were separated using Tukey's Honestly Significant Difference test. In both experiments, the pen was used as the experimental unit for analysis of all the parameters. Probability values of $\leqslant 0.05$ were considered as significant in both experiments.

Data generated in the second experiment was analyzed as a 2×2 factorial arrangement in a completely randomized design. Pens were considered the experimental unit for growth performance, and piglets were experimental units for measuring the digestibility of nutrients and all intestinal sampling. The main effects of bacteriophage cocktail and probiotics, and their interaction were determined by mixed procedure of SAS statistical program (SAS Institute Inc.). *P*-values $\leqslant 0.05$ were considered statistically significant.

Results

In Experiment 1, for phase I and II, pigs fed the diet supplemented with 1.0 and 1.5 g/kg bacteriophage cocktail showed reduced ($P < 0.05$) fecal score than the pigs fed the control diet (Table 2). In overall result, pigs fed the diet supplemented with 1.0 and 1.5 g/kg bacteriophage cocktail exhibited similar growth performance and had significantly better ($P < 0.05$) ADG than the pigs fed the control diet. Dietary treatments had no effects ($P < 0.05$) on G : F and ADFI of piglets.

In Experiment 2, in phase II of study, pigs fed the bacteriophage cocktail showed lower ($P < 0.05$; Table 3) fecal score. Pigs fed bacteriophage cocktail had greater ($P < 0.05$)

Table 2 *Effects of dietary levels of bacteriophage on growth performance and fecal score in weanling pigs (Experiment 1)*[1]

Bacteriophage (g/kg)	0 (Control)	1	1.5	SEM
Day 0 to day 7				
ADG (g)	308	334	330	7.67
ADFI (g)	437	446	440	5.24
G : F (g/kg)	705	761	749	15.51
Day 8 to day 21				
ADG (g)	392[b]	407[ab]	410[a]	4.52
ADFI (g)	544	549	545	12.31
G : F (g/kg)	721	741	756	18.96
Day 22 to day 35				
ADG (g)	470	485	480	8.51
ADFI (g)	742	744	749	5.27
G : F (g/kg)	634	653	640	11.57
Day 0 to day 35				
ADG (g)	404[b]	424[a]	421[a]	3.75
ADFI (g)	602	608	609	6.01
G : F (g/kg)	670	699	691	8.29
Fecal score				
Phase I (day 0 to day 7)	2.20	2.15	2.05	0.19
Phase II (day 8 to day 21)	1.84[a]	1.66[b]	1.51[b]	0.07
Phase III (day 22 to day 35)	1.50[a]	1.27[b]	1.29[b]	0.06

ADG = average daily gain; ADFI = average daily feed intake; G : F = gain to feed ratio.

[a,b]Values with different superscripts in the same row are significantly different ($P < 0.05$).

[1]Each mean based on six replicates.

Table 3 *Effects of dietary levels of bacteriophage on growth performance and occurrence of diarrhea in weanling pigs (Experiment 2)*

Bacteriophages (B)[1]	−		+			*P*-value		
Probiotics (P)[1]	−	+	−	+	SEM	P	B	P × B
Day 0 to day 7								
ADG (g)	317	329	326	338	9.19	0.214	0.33	1
ADFI (g)	431	440	438	443	8.17	0.419	0.581	0.801
G : F (g/kg)	737	748	745	763	21.34	0.551	0.691	0.918
Day 8 to day 21								
ADG (g)	381	398	404	422	8.66	0.066	0.012	0.954
ADFI (g)	548	539	549	556	8.21	0.904	0.253	0.341
G : F (g/kg)	696	738	736	760	12.47	0.091	0.113	0.627
Day 22 to day 35								
ADG (g)	438	459	463	449	10.48	0.226	0.112	487
ADFI (g)	758	736	745	741	8.71	0.134	0.631	0.318
G : F (g/kg)	577	625	623	634	14.42	0.051	0.058	0.214
Day 0 to day 35								
ADG (g)	391	408	412	418	7.64	0.072	0.026	0.747
ADFI (g)	609	598	605	608	6.75	0.521	0.619	0.354
G : F (g/kg)	643	683	681	694	9.42	0.018	0.028	0.314
Fecal score								
Phase I (day 0 to day 7)	1.82	1.85	1.91	1.89	0.11	0.565	0.599	0.883
Phase II (day 8 to day 21)	1.77	1.54	1.41	1.36	0.09	0.121	0.005	0.304
Phase III (day 22 to day 35)	1.59	1.43	1.39	1.35	0.09	0.239	0.125	0.526

ADG = average daily gain; ADFI = average daily feed intake; G : F = gain to feed ratio.
[1]0.10% bacteriophage cocktail and 0.30% probiotics.

Table 4 *Effects of dietary levels of bacteriophage on apparent total tract digestibility (%) of nutrients in weanling pigs (Experiment 1)*

Bacteriophage (g/kg)	0 (Control)	1	1.5	SEM
Day 7				
DM	82.96	82.74	83.67	0.47
GE	82.39	81.65	82.26	0.53
CP	79.36	78.73	78.56	0.48
Day 21				
DM	84.55	85.03	84.17	0.49
GE	82.31	83.71	83.89	0.52
CP	82.12[b]	83.26[ab]	83.67[a]	0.42
Day 35				
DM	83.05[b]	84.95[a]	84.33[a]	0.46
GE	83.59	83.17	83.45	0.53
CP	82.49	82.33	82.11	0.62

DM = dry matter; GE = gross energy.
[a,b]Values with different superscripts in the same row are significantly different ($P < 0.05$).

ADG in phase II and in overall result. Moreover, the overall G : F of piglets fed the bacteriophages or probiotics diets were significant ($P < 0.05$).

In Experiment 1, pigs fed diets supplemented with 1.0 and 1.5 g/kg bacteriophage cocktail exhibited similar ATTD of nutrients ($P > 0.05$) at day 7. However, digestibility of CP was significantly higher ($P < 0.05$) only in the highest level of bacteriophage cocktail (1.5 g/kg feed) than the piglets fed the control diet at day 21. Bacteriophage treatment group had significantly greater ($P < 0.05$; Table 4) ATTD of DM at day 35. In Experiment 2, the digestibility of nutrients was not significantly different among the groups (Table 5).

In Experiment 1, pigs fed diets supplemented with 1.0 and 1.5 g/kg bacteriophage cocktail had greater ($P < 0.05$; Table 6) ileal *Lactobacillus* spp. populations and fewer coliform and *Clostridium* spp. colonization (ileum and cecum) than that of pigs fed the control diet. Cecal *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* spp. and coliform populations were similar among pigs fed diet supplemented with 1.0 and 1.5 g/kg bacteriophage cocktail.

In Experiment 2, pigs fed probiotics had greater ($P < 0.05$; Table 7) ileal *Lactobacillus* spp. populations, however, coliform, *Clostridium* spp. and *Bifidobacterium* spp. numbers were similar in both ileum and cecum. Fewer ($P < 0.05$) coliform (cecum) and *Clostridium* spp. (cecum and ileum) populations were observed in pigs fed bacteriophage cocktail.

In Experiment 1, pigs fed the diet supplemented with 1 or 1.5 g/kg bacteriophage product had greater ($P < 0.05$) villus height, but there was no effect of bacteriophages or probiotics on intestine crypt depth (Table 8).

In Experiment 2, pigs fed probiotics ($P = 0.051$) or bacteriophage treatment group ($P < 0.05$; Table 9) had greater villus height (duodenum). Pigs within the bacteriophage treatment group were found to have deeper crypts in the duodenum.

Discussion

In recent years, considerable efforts have been made for developing novel non-antibiotic feed additives which have the potential to improve the gut health, immunity and

Table 5 *Effects of dietary probiotics, bacteriophage or their combination on apparent total tract digestibility (%) of nutrients in weanling pigs (Experiment 2)*

Bacteriophages (B)[1]	−		+			*P*-value		
Probiotics (P)[1]	−	+	−	+	SEM	P	B	P × B
Day 7								
DM	83.58	83.17	83.33	83.50	0.62	0.841	0.946	0.642
GE	82.65	82.47	82.98	82.00	0.57	0.319	0.908	0.492
CP	78.90	78.85	78.61	78.75	0.57	0.943	0.742	0.874
Day 21								
DM	82.28	82.15	82.34	82.55	0.66	0.97	0.721	0.815
GE	80.23	80.33	80.75	80.78	0.64	0.919	0.463	0.959
CP	78.71	79.40	79.08	80.08	0.66	0.215	0.434	0.812
Day 35								
DM	80.75	81.13	81.3	81.65	0.72	0.618	0.47	0.981
GE	78.20	78.60	78.96	78.91	0.65	0.732	0.413	0.79
CP	76.83	77.05	77.90	77.73	0.62	0.968	0.172	0.759

DM = dry matter; GE = gross energy.
[1]0.10% bacteriophage cocktail and 0.30% probiotics.

Table 6 *Effect of dietary levels of bacteriophage in microflora (log_{10} cfu/g) in ileum and cecum content of weanling pigs (day 35; Experiment 1)*

Bacteriophage (g/kg)	0 (Control)	1	1.5	SEM
Ileum				
Lactobacillus spp.	8.42[b]	8.73[a]	8.68[a]	0.08
Coliforms	6.42[a]	6.11[b]	6.05[b]	0.06
Clostridium spp.	7.46[a]	7.28[b]	7.16[b]	0.04
Bifidobacterium spp.	8.53	8.73	8.62	0.16
Cecum				
Lactobacillus spp.	8.15	8.26	8.18	0.16
Coliforms	5.83	6.01	5.95	0.16
Clostridium spp.	7.50	7.24	7.17	0.18
Bifidobacterium spp.	8.34	8.61	8.59	0.27

[a,b]Values with different superscripts in the same row are significantly different ($P < 0.05$).

performance of pigs (Jin *et al*., 2009; Choi *et al*., 2011b; Yoon *et al*., 2012 and 2014). Among various alternatives, bacteriophages have received attention due to their natural antimicrobial properties and lower propensity for the development of bacterial resistance (Gebru *et al*., 2010; Wittebole *et al*., 2014; Kim *et al*. 2014b). The present study demonstrates the effects of bacteriophage cocktail, fermented probiotic product and their combination on performance and gut health of weanling pigs.

Results from the current study demonstrate that dietary bacteriophage cocktail and probiotics both improve G : F (Experiment 2) and ATTD of DM (Experiment 1) but the combination of bacteriophage and probiotics did not show a superior interaction. Our results are in agreement with Kim *et al*. (2014b), who observed improvement in growth performance and ATTD of nutrients in pigs fed diets supplemented with bacteriophage and probiotics. Similarly, Gebru *et al*. (2010) reported improvement in of overall ADG and G : F of *Salmonella* challenged pigs fed diets supplemented with 3×10^9 pfu/kg diet *S. typhimurium* bacteriophage. In contrast to the report by Kim *et al*. (2014b) and present results, Yan *et al*. (2012) observed that dietary supplementation with anti-*Salmonella* bacteriophage had no effect on ADG and G : F of growing pigs. These variations in the results are likely associated with variations in age of pigs, level and type of bacteriophage supplemented, health status within herds, farm hygiene, diet composition, feed forms and interactions with other dietary feed additives. Phages probably pass gastro-intestinal tract safely because they are able to survive in an acidic environment, however, pH = 6 to 7 is the optimum range for most of them to show the highest efficiency. Lu *et al*. (2003) reported that 24 phage isolates were obtained in fermentation tanks with sauerkraut (pH < 3.5) after 60 days. Yan *et al*. (2012) reported that growing pigs fed diets supplemented with anti-*Salmonella* bacteriophage had greater digestibility of DM, which is in agreement with our results (Experiment 1, phase 3). The results of our second study indicated the growth promoting of 1.0 g/kg diet bacteriophage cocktail and showed there were not synergistic effects of combining the bacteriophage cocktail with the fermented probiotic product. Similarly, Kim *et al*. (2014b) working with growing pigs reported that there were no synergistic effects of combining the bacteriophages with the probiotics. In the present trials, improved ADG of the pigs with dietary inclusion of bacteriophages in both Experiments 1 and 2 might be associated with a relative reduction of coliforms and *Clostridium* spp. or improved intestinal morphology. Reduced fecal score as a sign of incidence of diarrhea in weaning pigs fed the diet supplemented with bacteriophages reported in the present study is in agreement with Jamalludeen *et al*. (2009) who observed that a combination of three bacteriophages could prevent *E. coli* induced diarrhea in weaned pigs. Weaned piglets seem to be more susceptible to intestinal infection by

Table 7 *Effect of dietary probiotics, bacteriophage or their combination on small intestine microbiota (Experiment 2; day 35)*

Bacteriophages (B)[1]	−		+			*P*-value		
Probiotics (P)[1]	−	+	−	+	SEM	P	B	P × B
Ileum								
Lactobacillus spp.	8.51	8.78	8.63	8.81	0.09	0.026	0.385	0.627
Coliforms	6.38	6.21	6.02	5.85	0.16	0.312	0.038	0.971
Clostridium spp.	7.31	7.46	7.11	7.07	0.13	0.672	0.035	0.493
Bifidobacterium spp.	8.47	8.96	8.79	8.56	0.19	0.511	0.816	0.073
Cecum								
Lactobacillus spp.	8.2	8.58	8.48	8.66	0.24	0.253	0.472	0.674
Coliforms	6.31	6.19	5.99	5.81	0.24	0.536	0.159	0.901
Clostridium spp.	7.64	7.29	7.17	6.88	0.18	0.1	0.029	0.864
Bifidobacterium spp.	8.28	8.73	8.47	8.64	0.19	0.133	0.824	0.492

[1]0.10% bacteriophage cocktail and 0.30% probiotics.

Table 8 *Effect of dietary levels of bacteriophage on small intestinal morphology of weanling pigs (day 35; Experiment 1)*

Bacteriophage (g/kg)	0 (Control)	1	1.5	SEM
Villus height (μm)				
Duodenum	496[b]	528[a]	536[a]	8.21
Jejunum	551[b]	577[a]	576[a]	8.40
Ileum	440	447	442	9.66
Crypt depth (μm)				
Duodenum	312	334	326	11.12
Jejunum	361	377	361	9.89
Ileum	263	256	250	10.17
Villus height/crypt depth				
Duodenum	1.59	1.56	1.64	0.09
Jejunum	1.51	1.53	1.6	0.12
Ileum	1.67	1.75	1.77	0.13

[a,b]Values with different superscripts in the same row are significantly different ($P < 0.05$).

pathogens such as coliforms and *Clostridium* spp. Diarrhea occurring after the weaning period can be caused in different situations including the presence of *E. coli* (Hopwood *et al*., 2005). The lower colonization of coliforms and *Clostridium* spp. can be the main reason for lower fecal score.

In the present trials, pigs fed diets supplemented with probiotics had higher *Lactobacillus* spp. in ileum. There are numerous studies about probiotics which show higher lactobacillus colonization in intestine (Choi *et al*., 2011a and 2011b; Baker *et al*., 2013). We had hypothesized an interaction between bacteriophages and probiotics, because bacteriophages have the ability of eliminating pathogens and give the opportunity to *Lactobacillus* spp. from probiotics to proliferate in a less competitive environment. Furthermore, *Lactobacillus* genus are the most closely studied group of probiotics that facilitate the growth of certain microbes while inhibiting the growth of others particularly enterobacteria such as *Salmonella* and *E. coli* mostly by the reduction in pH due to anaerobic fermentation in the intestine (Kenny *et al*., 2011). However, probiotics in the current study did not affect the colonization of pathogens. Fewer coliforms in ileum was the result of using bacteriophages in the diet, but the combination of bacteriophages and probiotics were not significantly effective. Kim *et al*. (2014a) observed reduced *Clostridium Perfrigensis* shedding score in broiler chickens fed diets supplemented with a bacteriophage cocktail including an anti-*Clostridium* bacteriophage. In contrast to the present results, Kim *et al*. (2014b) observed no additional benefits of combination of bacteriophage and probiotics on fecal *Lactobacillus* spp. and coliforms populations. It has been well established that antimicrobial feed additives beneficially affect the host animal by improving its intestinal balance (Fuller, 1989) and creating gut micro-ecological conditions that suppress harmful microorganisms like coliforms and by favoring beneficial microorganisms like *Lactobacillus* spp. (Heyman and Menard, 2002; Choi *et al*., 2011a; Yoon *et al*., 2012 and 2014). In the current experiment, bacteriophages had the most notable effects against *Clostridium* spp. as the main cause of necrotic enteritis, since their population dramatically decreased in ileum of the first experiment and in both Ileum and cecum in the second experiment. In a previous study, it was showed that bacteriophage proteins were capable of lysing *C. perfringens* and could be expressed in yeast and added as lysates to animal feed to reduce the bacterium to improve health and food safety for monogastrics animals during production (Miller *et al*., 2010). The host-specificity role of bacteriophages (Endersen *et al*., 2014) can be considered as a positive feature of phage therapy because they only infect bacteria which have the membrane receptor. Tail penetration through cell walls degraded enzymatically drives insertion of phage DNA into the cytoplasm of the host to encode specific enzymes and proteins by the phages genome in order to lyse host cell (Endersen *et al*., 2014). Another experiment with humans showed that prophylactic phage therapy of *Clostridium difficile* infection can considerably reduce the burden of *C. difficile* vegetative cells and caused a clinically relevant decrease in toxin production (Meader *et al*., 2013). Results obtained herein provide new information regarding potential of bacteriophage or probiotics for improving intestinal microbiota of weanling pigs.

Table 9 *Effect of dietary probiotics, bacteriophage or their combination on small intestinal morphology of weanling pigs (Experiment 2; day 35)*

Bacteriophages (B)[1]	−		+			*P*-value		
Probiotics (P)[1]	−	+	−	+	SEM	P	B	P × B
Villus height (μm)								
Duodenum	487	542	547	556	11.24	0.053	0.012	0.203
Jejunum	567	592	564	607	10.28	0.115	0.814	0.218
Ileum	431	438	448	452	9.25	0.69	0.508	0.407
Crypt depth (μm)								
Duodenum	313	330	341	348	9.68	0.233	0.03	0.617
Jejunum	349	351	325	339	11.37	0.481	0.136	0.624
Ileum	252	255	266	260	8.94	0.868	0.285	0.62
Villus height/crypt depth								
Duodenum	1.56	1.64	1.6	1.6	0.058	0.466	0.72	0.843
Jejunum	1.62	1.69	1.74	1.79	0.061	0.689	0.118	0.751
Ileum	1.71	1.72	1.68	1.74	0.089	0.899	0.841	0.756

[1]0.10% bacteriophage cocktail and 0.30% probiotics.

The changes in small intestinal morphology and in particular the villus height and crypt depth of duodenum, jejunum and ileum are indicative of gut health and digestive capacity of pigs. Increased villus height is directly correlated with an increased epithelial turnover (Fan *et al*., 1997), and longer villi correlate with the activation of cell mitosis (Samanya and Yamauchi, 2002). In the present study, dietary supplementation with bacteriophages and probiotics ($P = 0.053$) reported increased villus height of duodenum. However, there were no additional beneficial effects of combining bacteriophages and probiotics. Previous studies in our laboratory on pigs and broiler reported improved intestinal morphology with dietary supplementation with fermented probiotics product (Choi *et al*., 2011a and 2011b; Kim *et al*., 2012). Mourao *et al*. (2005) reported that a decreased number of pathogenic bacteria in the gut may improve proliferation of epithelial cells to build villus and thus enhance intestinal morphology. In general there is a void of information regarding the effect of feeding bacteriophages on the histomorphology of small intestine of weaning piglets. In the present study, dietary bacteriophages and probiotic produced a relative improvement in villus size; however it was a non-uniform alteration in the individual intestinal segments.

Conclusion

In conclusion, the results obtained in the present study indicate that the bacteriophages are believed to influence the colonization of coliforms and *Clostridium* spp. in ileum, it can be postulated that fewer mentioned pathogens may have helped improve intestinal morphology and the growth performance, however, there were no interactive effects between bacteriophages and probiotics.

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