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Dietary bacteriophages as an alternative for zinc oxide or organic acids to control diarrhoea and improve the performance of weanling piglets

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ABSTRACT: In this study, the antibacterial substances ZnO, organic acids and a bacteriophage cocktail were added to the diet of weanling pigs to evaluate the effects on gut health. Dietary treatments were basal diet without any supplementation (Control) and basal diet either supplemented with 0.34% (2500 ppm) ZnO, 0.20% organic acids or with 0.10% bacteriophage cocktail. Faecal score was decreased in ZnO and bacteriophage cocktail treatments. The total number of ileal anaerobic bacteria, *Bifidobacterium* spp. and *Lactobacillus* spp. were higher in ZnO, bacteriophage cocktail and organic acids, while ileal coliforms and caecal *Clostridium* spp. were decreased in comparison to Control. Faecal coliforms (Day 7 and Day 21) and *Clostridium* spp. (Day 21 and Day 35) were lower in bacteriophage cocktail. The gain to feed ratio was improved in all supplemented groups. The digestibility of dry matter was increased at the end of the experiment in all supplemented groups, while that of crude protein was increased only at Day 21 in bacteriophage cocktail. Duodenal villus height was increased in ZnO and bacteriophage cocktail. Bacteriophage cocktail also showed a greater villus height in the small intestine. Supplementation of bacteriophage cocktail in weaning pig diets resulted in better growth performance, digestibility and gut development compared to Control, and thus, it can be concluded that its effects are comparable to ZnO or organic acids supplementation.

Keywords: growth performance; intestinal morphology; microbiota; faecal score; coliforms; *Clostridium*

A lag in the growth during weaning is a crucial issue affecting the profitability of the swine industry. The susceptibility of weaning piglets to infection with several species of bacteria, such as *Escherichia coli* and *Clostridium perfringens*, may cause diseases resulting in decreased growth and in some cases even death (Walsh et al. 2003). Previously, antibiotics were generally included in diets to overcome post-weaning problems, maintain gut health, enhance performance and to protect against diseases such as diarrhoea (Partanen and Morz 1999). However, antibiotic resistance (Simon 2005) had become a serious problem in the pig industry, and thus, the utilisation of antibiotics as growth promoters for livestock is now prohibited. For this reason, alternatives such as probiotics, prebiotics, organic acids (OA), ZnO, antimicrobial peptides etc., have been tested as replacements for antibiotics.

Zinc is an essential element and its recommended intake for growing pigs is 50–100 mg/kg dry matter (NRC 2012); however, higher levels of ZnO enhance growth rates and are well known to prevent or alleviate diarrhoea in young pigs and to improve growth performance (Carlson et al. 1999; Hollis et al. 2005), by mechanisms which are not well documented. High levels of ZnO were thus widely used in the pig industry but environmental concerns have forced the European Union to pass legislation to restrict the use of ZnO to a maximum of 150 mg/kg (European Community 2003) of feed,

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which is much less than the anti-diarrhoeal dosage (more than 2000 mg/kg). Two years later, South Korea also set an upper limit of 2500 mg/kg ZnO in feed but this value is still approximately 20 times more than a pig's growth requirement (MAFRA 2014). Therefore, it is to be expected that ZnO will soon be banned at pharmacological levels in many countries due to concerns regarding pollution of land and water with excretions from manure.

OA have previously been used in weanling pigs and are known to improve nutrient digestibility and growth performance (Upadhaya et al. 2014), and to modify the intestinal microflora (Kim et al. 2005). It has been reported that OA added to the diets of young chicks altered the microbial population of the small intestine (Cengiz et al. 2012). These substances have been tested as alternatives to antibiotics (Mroz 2005), and their antimicrobial efficiency varies according to their concentration and pH (Chaveerach et al. 2002). After the restrictions set on ZnO levels, the use of OA has increased as part of efforts to control the incidence of diarrhoea in the European Union, and although the low pH of OA is also harmful for feed processing facilities in the long-term, these substances remain good candidates for the control of diarrhoea.

The most recent development regarding in-feed antimicrobials in the pig industry is the use of bacteriophages (BA). Bacteriophages are viral entities that multiply inside bacteria by using the latter's biosynthetic machinery and act as natural predators of their hosts. Their inherent antimicrobial properties make them ideal candidates for controlling bacterial levels (Wittebole et al. 2014), and their use carries a lower risk of the development of bacterial resistance. Previous studies reported decreases in the concentration of pathogenic bacteria and prevention of *E. coli*-induced diarrhoea in weaned pigs (Jamalludeen et al. 2009). Therefore, the present experiment was designed to study the growth-promoting and antimicrobial effects of BA supplementation in the diets of weanling pigs in comparison with ZnO and OA.

Therefore, these experiments were conducted to compare the effects of ZnO, OA or BA as different inhibitors of diarrhoea with different mode of action, on diarrhoea incidence, microbial population, gut morphology, growth performance and nutrient digestibility of weanling pigs. We hypothesised that the supplementation of BA alone, without ZnO or OA, would be beneficial in diets for improving the performance of pigs under normal conditions.

MATERIAL AND METHODS

The experiment was conducted at the facilities of the Kangwon National University farm and was approved by the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Republic of Korea. ZnO was procured from TMC Co. Ltd. The mixture of OA containing 25% lactic acid, 15% formic acid and 15% citric acid, was procured from Eunjin Bio International Co. Ltd. The BA combination that was used in this study was obtained from a commercial feed company (CTC Bio Inc., Seoul, Republic of Korea). It contained a cocktail of BA: 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 with 109 plaque-forming units per gram (pfu/g) BA.

Animals, diets and managements. A total of 200 piglets (Landrace × Yorkshire × Duroc) of mixed sex were randomly allotted to four treatment groups on the basis of initial body weight (average weight 7.27 ± 0.26 kg). There were five replicate pens in each treatment with 10 pigs per pen. Dietary treatments were, basal diet without any supplementation (Control), and basal diet either supplemented with 0.34% ZnO (2500 ppm ZnO), 0.20% OA or 0.10% BA cocktail. The experimental diets exceeded the nutrient requirements as suggested by NRC (2012) and were fed in meal form in three phases (Phase I from Day 0 to 7, Phase II from Day 8 to 21, and Phase III from Day 22 to 35) for a total of 35 days. The pigs used in the study were housed in partially slatted and concrete floor pens of 2.80 × 5.00 m. All the pens were equipped with a self-feeder and nipple drinker to allow *ad libitum* access to feed and water.

Sampling and measurements. Pigs were weighed individually, and feed consumption was calculated at the end of each experiment to calculate average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G : F). To determine the effect of different treatments on the apparent total tract digestibility (ATTD), chromic oxide (2.50 g/kg) was added to each diet and faecal grab samples were collected during the last four days of each phase of the experiment to determine the ATTD of dry matter (DM), gross energy (GE) and crude protein (CP). The faecal samples were then pooled within each pen, dried in a forced air oven at 60 °C for 72 h, ground in a Wiley mill

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(Thomas Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) using a 1-mm screen and used for chemical analysis. To evaluate the faecal microbial content, on the final day of experiment, i.e. at Day 35, fresh faecal samples from two pigs in each pen were collected and stored on ice before measurements of the faecal bacterial population.

To study the effect of the diets on ileal and caecal digesta, two pigs per pen of average body weight from each group were euthanized by electrocution at Day 35. The ileum and caecum digesta were collected in sterile plastic bottles for microbial analysis and were immediately placed on ice until analyses.

Chemical and microbial analyses. Analysis for each sample was done in triplicate for DM (Method 930.15), CP (Method 990.03), ash (Method 942.05), Ca and P (Method 985.01) according to the methods of the AOAC (2007). GE of diets and excreta were measured using a bomb calorimeter (Model 1261, Parr Instrument Co., Molin, USA), while chromium concentrations were determined with an automated spectrophotometer (Shimadzu, Japan) according to the procedure described by Fenton and Fenton (1979). To determine the total anaerobic bacteria (tryptic soy agar), *Lactobacillus* spp. (using MRS agar + 0.200 g/l NaN3 + 0.500 g/l L-cystine hydrochloride monohydrate), *Bifidobacterium* spp. (MRS-NPNL: MRS agar + nalidixic acid, paromomycin + neomycin sulphate + lithium chloride), *Clostridium* spp. (TSC agar) and coliforms (violet red bile agar) were used. Anaerobic conditions during the assay of total anaerobic bacteria and *Clostridium* spp. were created by using a gas pack anaerobic system (BBL, No. 260678, Difco, Detroit, USA). The tryptic soy agar (No. 236950), MRS agar (No. 288130) and violet red bile agar (No. 216695), were purchased from Difco Laboratories (Detroit, USA), and TSC agar (CM0589) was purchased from Oxoid (Hampshire, UK). The bacterial concentrations were transformed (log) before statistical analysis.

Small intestinal morphology. For each intestinal sample, three cross-sections were prepared after staining with azure A and eosin using standard paraffin embedding procedures. A total of 10 intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section. The measurement of villus height was done from the tip of the villi to the villus crypt junction, while the crypt depth was defined as the depth of the invagination between adjacent villi and villus width was measured until the midpoint of the villus. All morphological measurements (villus height and crypt depth) were made in 10-µm increments by using an image processing and analysis system (Optimus software version 6.5, Media Cybergenetics, North Reading, USA).

Diarrhoea incidence. The incidence of diarrhoea was measured by scoring the faeces as zero (normal), one point (soft faeces), two points (mild diarrhoea), three points (severe diarrhoea) in all the experiments. The overall cumulative incidence of diarrhoea was measured daily at 09:00 h for five weeks and the final diarrhoea incidence was determined as the average of the scores.

Statistical analysis. Data generated in the present study were subjected to statistical analysis using the GLM-one-way analysis of variance test using the SAS statistical software package version 8.2 (SAS Inst. Inc., Cary, USA). The pen was defined as the experimental unit for the analysis of growth performance parameters, whereas the individual pig was used as the experimental unit for analysis of faecal score, microbial population and intestinal morphology parameters. When significant differences ($P < 0.05$) were noted among treatment means, they were separated using Tukey's test.

RESULTS

Faecal score

The faecal score is presented in Table 1. There was no variation ($P > 0.05$) in the faecal score in Phase I (Day 0–7) and Phase II (Day 7–21); however, it was lower in BA and ZnO groups in both

Table 1. Effect of dietary treatments on faecal scores in weaning pigs

	Control	ZnO	OA	BA	SEM	*P*-value
Phase I (Day 0–7)	1.74	1.47	1.58	1.62	0.07	0.105
Phase II (Day 7–21)	1.64	1.23	1.34	1.22	0.11	0.074
Phase III (Day 21–35)	1.46^{a}	1.09^{b}	1.28^{ab}	1.06^{b}	0.09	0.025
Overall (Day 0–35)	1.63^{a}	1.24^{b}	1.35^{ab}	1.26^{b}	0.06	0.004

BA = bacteriophages, OA = organic acids

$^{a–b}$Values with different superscripts in the same row differ significantly ($P < 0.05$)

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Phase I (P = 0.105) and Phase II (P = 0.074). From then the diarrhoea incidence decreased (P < 0.05) in Phase III (Day 21–35) in ZnO and BA treatments. The overall results also showed a lower faecal score in ZnO and BA groups (P < 0.05) compared with Control.

Intestinal and faecal microbiota composition

The results of assaying intestinal and faecal microbial populations are presented in Tables 2 and 3. All supplementations increased the number of total anaerobic bacteria and *Bifidobacterium* spp. in ileum. BA and OA groups showed a lower number of caecal *Clostridium* spp. and ileal coliforms compared to Control. Similarly, determination of microbial populations in faeces revealed that the number of coliforms was lower (P < 0.05) in OA and BA at Day 7, and lower in BA at Day 21. *Clostridium* spp. colonisation was decreased in the BA-supplemented group at Day 21 and 35. Total faecal anaerobic bacteria were significantly different (Table 3) with a P-value of 0.035. The population of *Lactobacillus* spp. in faeces did not differ among the treatments at Day 7 and 21; however, the BA group showed a higher extent of *Lactobacillus* spp. colonisation at the end of the experiment.

Table 2. Effect of dietary treatments on intestinal microbial populations(log CFU/g) in weaning pigs (Day 35)

	Control	ZnO	OA	BA	SEM	P-value
Ileum						
Total anaerobic bacteria	6.67^{b}	7.28^{a}	7.33^{a}	7.32^{a}	0.13	0.012
Bifidobacterium spp.	8.36^{b}	8.93^{a}	8.86^{a}	8.89^{a}	0.12	0.015
Lactobacillus spp.	8.11^{b}	8.45^{ab}	8.65^{a}	8.61^{a}	0.09	0.004
Clostridium spp.	5.74	5.52	5.57	5.24	0.15	0.185
Coliforms	6.15^{a}	5.78^{b}	5.67^{bc}	5.35^{c}	0.07	< 0.001
Cecum						
Total anaerobic bacteria	8.49	8.61	8.69	8.64	0.11	0.621
Bifidobacterium spp.	8.25	8.74	8.64	8.72	0.14	0.084
Lactobacillus spp.	8.37	8.38	8.35	8.32	0.09	0.979
Clostridium spp.	7.37^{a}	7.21^{ab}	6.89^{bc}	6.61^{c}	0.10	0.001
Coliforms	6.42	6.12	6.17	6.19	0.09	0.114

BA = bacteriophages, OA = organic acids

$^{a–c}$Values with different superscripts in the same row differ significantly (P < 0.05)

Performance

The test was conducted in three phases and is presented in Table 4. The basal diet supplemented with the BA cocktail consistently resulted in higher (P < 0.05) ADG in Phases I and II in comparison to Control. Supplementation with either ZnO or OA did not have any effect on the ADG in any of the three phases. The overall value was also higher (P < 0.05) in the BA group compared with Control. ADFI did not differ (P >0.05) among the treatments. Adding ZnO or BA to the diet improved G : F in

Table 3. Effect of dietary treatments on faecal microbial populations (log CFU/g) in weaning pigs

	Control	ZnO	OA	BA	SEM	P-value
Day 7						
Total anaerobic bacteria	8.57	9.07	9.05	9.09	0.15	0.078
Bifidobacterium spp.	8.06	8.47	8.19	8.45	0.14	0.120
Lactobacillus spp.	8.05	8.50	8.60	8.59	0.14	0.055
Clostridium spp.	8.53	8.29	8.49	8.21	0.12	0.201
Coliforms	7.05^{a}	6.93^{ab}	6.60^{b}	6.58^{b}	0.10	0.009
Day 21						
Total anaerobic bacteria	8.94	9.42	9.41	9.35	0.12	0.035
Bifidobacterium spp.	8.23^{b}	8.65^{ab}	8.80^{a}	8.72^{ab}	0.18	0.024
Lactobacillus spp.	7.73	7.71	8.26	8.17	0.18	0.116
Clostridium spp.	7.96^{a}	8.03^{a}	7.47^{ab}	7.34^{b}	0.14	0.009
Coliforms	6.98^{a}	6.52^{a}	6.57^{ab}	6.32^{b}	0.12	0.018
Day 35						
Total anaerobic bacteria	9.01	9.50	9.57	9.51	0.15	0.070
Bifidobacterium spp.	8.50	8.76	8.94	8.87	0.20	0.449
Lactobacillus spp.	8.07^{b}	8.05^{b}	8.49^{ab}	8.76^{a}	0.12	0.003
Clostridium spp.	8.27^{a}	7.73^{ab}	7.77^{ab}	7.62^{b}	0.15	0.045
Coliforms	6.81	6.70	6.48	6.40	0.18	0.376

BA = bacteriophages, OA = organic acids

$^{a–b}$Values with different superscripts in the same row differ significantly (P < 0.05)

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Table 4. Effect of dietary treatments on the growth performance of weanling pigs

	Control	ZnO	OA	BA	SEM	*P*-value
Phase I (Day 0–7)						
ADG (g)	340[b]	366[ab]	361[ab]	376[a]	8.04	0.044
ADFI (g)	425	420	432	434	5.38	0.247
G : F	0.80[b]	0.874[a]	0.836[ab]	0.867[a]	0.01	0.009
Phase II (Day 7–21)						
ADG (g)	398[b]	426[ab]	428[ab]	433[a]	7.61	0.027
ADFI (g)	645	634	639	634	4.93	0.367
G : F	0.617[b]	0.672[a]	0.670[a]	0.683[a]	0.01	0.002
Phase III (Day 21–35)						
ADG (g)	510	512	518	529	9.88	0.529
ADFI (g)	859	857	860	863	6.04	0.884
G : F	0.594	0.597	0.602	0.613	0.01	0.694
Overall (Day 0–35)						
ADG (g)	431[b]	448[ab]	451[ab]	460[a]	4.57	0.005
ADFI (g)	687	680	686	686	3.28	0.508
G : F	0.644[b]	0.683[a]	0.676[a]	0.692[a]	0.01	0.004

ADFI = average daily feed intake, ADG = average daily gain, BA = bacteriophages, G : F = gain to feed ratio, OA = organic acids

[a–b]Values with different superscripts in the same row differ significantly ($P < 0.05$)

Phases I and II. The G : F ratio was greater in Phase II when OA was supplemented in the diet ($P < 0.01$). The overall G : F ratio (Day 0–35) was significantly greater in all supplemented groups compared to Control.

Nutrient digestibility

The effect of different treatments on nutrient digestibility is presented in Table 5. Dry matter digestibility in all the treatment groups (ZnO, OA and BA) was higher ($P < 0.05$) at Day 35 (Phase III), while no differences were observed in the GE compared with Control. At Day 21 (Phase II), the CP was higher in the BA group compared to Control but was not significantly different ($P > 0.05$) in Phases I and III.

Intestinal morphology

Supplementation with ZnO increased villus height in the duodenum ($P < 0.05$; Table 6), but not in the jejunum and ileum. Supplementation with BA increased villus height in the duodenum and jejunum; however, there was no difference in ileal villus height. No significant differences were found in duodenal, jejunal and ileal crypt depths or in villus height to crypt depth ratios (VH : CD).

Table 5. Effect of dietary treatments on apparent total tract digestibility in weaning pigs

	Control	ZnO	OA	BA	SEM	*P*-value
Day 7						
DM	82.10	83.14	83.01	83.20	0.43	0.281
GE	80.55	81.01	80.97	80.95	0.39	0.828
CP	75.38	75.69	75.73	75.75	0.37	0.882
Day 21						
DM	80.14	80.95	80.67	80.81	0.32	0.356
GE	79.28	79.55	79.42	79.55	0.28	0.89
CP	73.57[b]	74.07[ab]	74.06[ab]	75.83[a]	0.50	0.036
Day 35						
DM	78.52[b]	79.56[a]	79.87[a]	79.84[a]	0.21	0.002
GE	77.84	78.13	78.27	78.34	0.34	0.756
CP	73.02	73.39	73.59	73.57	0.27	0.461

BA = bacteriophages, CP = crude protein, DM = dry matter, GE = gross energy, OA = organic acids

[a–b]Values with different superscripts in the same row differ significantly ($P < 0.05$)

Table 6. Effect of dietary treatments on small intestinal morphology in weaning pigs (Day 35)

	Control	ZnO	OA	BA	SEM	*P*-value
Villus height (μm)						
Duodenum	499[b]	532[a]	496[b]	529[a]	7.02	0.005
Jejunum	551[b]	574[ab]	568[ab]	585[a]	6.67	0.020
Ileum	429	451	445	457	11.37	0.354
Crypt depth (μm)						
Duodenum	313	324	321	308	5.41	0.182
Jejunum	316	324	326	326	4.52	0.409
Ileum	260	272	267	268	3.41	0.139
VH : CD						
Duodenum	1.59	1.64	1.56	1.72	0.08	0.161
Jejunum	1.74	1.77	1.74	1.79	0.04	0.756
Ileum	1.65	1.66	1.67	1.71	0.05	0.581

BA = bacteriophages, OA = organic acids, VH : CD = villus height to crypt depth ratio

[a–b]Values with different superscripts in the same row differ significantly ($P < 0.05$)

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DISCUSSION

Enhancing normal gut microflora by targeting intestinal pathogens through non-antibiotic approaches can improve the gut health, immunity, and performance of pigs and poultry (Suryanarayana et al. 2012; Yoon et al. 2012). Among various non-antibiotic alternatives, OA (Lambert and Stratford 1999; Suryanarayana et al. 2012) and zinc sources (Hollis et al. 2005) have been used previously. Further, BA have received increasing attention due to their natural antimicrobial properties and the lower propensity for the development of bacterial resistance (Gebru et al. 2010; Wittebole et al. 2014). The transient period in which weaning pigs progress from lactation to a solid diet is critical and often results in small-intestinal atrophy and dysfunction in piglets (Wu et al. 1996). The synthesis and absorption of semi-essential amino acids is thus reduced and is one of the major factors causing diarrhoea in neonates (Pluske et al. 1997). In the present study, there was a significant decrease in the diarrhoea incidence in the ZnO and BA groups. Inclusion of high levels of ZnO in the diet is beneficial for maintaining the normal morphology of the gastrointestinal tract in weaning pigs (Carlson et al. 1999; Li et al. 2001). In this study, ZnO increased duodenal villus height. This suggests that ZnO protects the small intestine from weaning-associated damage, which could be the reason behind the decreased diarrhoea incidence. Supplementation of piglet diets with a high ZnO dose (2.5 g/kg diet) or a lower ZnO dose (1.7 g/kg diet) supplemented with sodium humate (20.0 g/kg diet) was in both cases shown to be effective in the prophylaxis of post-weaning diarrhoea (Trckova et al. 2015). Supplementing a pea protein-based diet with 2880 mg of Zn/kg not only reduced the incidence of diarrhoea, but also expedited the recovery of piglets in this treatment group to within four days of *E. coli* administration; by contrast, piglets fed a diet without ZnO continued to shed *E. coli* K88 and had severe diarrhoea (Owusu-Asiedu et al. 2003). The decrease of diarrhoea in response to BA treatment might be due to the use of *E. coli* phage in the BA cocktail as previously, Jamalludeen et al. (2009) suggested that inclusion of phages could prevent *E. coli*-induced diarrhoea in weaned pigs. *E. coli* is recognised as a common pathogen associated with post-weaning diarrhoea in piglets (Melin et al. 2004). *Clostridium* spp. are Gram-positive, anaerobic, spore-forming bacilli that have the ability to spread indirectly via the faecal-oral route through spores left on surfaces. They produce two cytotoxins, which bind to receptors on intestinal epithelial cells, leading to inflammation and diarrhoea (Hookman and Barkin 2009). The toxins loosen the junctions of the epithelial cells allowing the toxins to penetrate (Starr 2005). In the current experiment, the BA cocktail as well as ZnO controlled the population of *Clostridium* spp. in the ileum, probably contributing to the improved faecal score.

Many genetic, physiological and environmental factors govern gut development in young animals. Generally, nutrients are hydrolysed by digestive enzymes in the small intestine and are fermented in the large intestine by anaerobic bacteria to yield short-chain fatty acids which can be resorbed and used by the host (Cummings and Macfarlane 1991). In the present study, the ileal populations of "friendly" bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. was found to be higher in the BA group. Simultaneously, the colonisation of caecal and faecal *Clostridium* spp. and coliforms was decreased in the BA group. This is in line with previous studies in which increases in the concentration of BA in pig diets resulted in increased numbers of *Bifidobacterium* spp. and *Lactobacilli* and decreased numbers of *Clostridium* spp. and coliforms (Yan et al. 2012; Kim et al. 2014). The increase in the population of "friendly" bacteria is likely due to the reduction in the *E. coli* and *Costridium* spp. Populations, which in turn might be due to the presence of related phages present in the BA cocktail. These phages may have been pathogenic to the *E. coli* and *Costridium* spp. and thus, might have provided a better ecosystem for the development of "friendly" bacteria such as *Lactobacillus* (Yan et al. 2012). OA are well known for both their bacteriostatic and bactericidal properties. They penetrate bacterial cells in either their non-ionized form and disrupt the normal physiology of the bacteria, or they become dissociated inside the bacterial cell to H^+ and anions (A^-) and reduce the internal pH of the bacteria (Lambert and Stratford 1999). In the present study, the populations of caecal *Clostridium* spp. and faecal coliforms (Phase I) were significantly reduced while the populations of ileal *Bifidobacterium* spp. and *Lactobacillus* spp. were considerably increased. These observa-

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tions could be due to the fact that coliforms and *Clostridium* are pH-sensitive and cannot tolerate the changes in pH caused by the OA, while bacteria such as *Lactobacilli* spp. and *Bifidobacterium* spp. are non-pH sensitive and can proliferate easily under these conditions (Gauthier 2002). The bactericidal effects of OA were not consistent across all periods.

In the present study, supplementation with ZnO and OA had no effect on the overall ADG but BA supplementation in the diets of weanling pigs consistently increased the ADG. There were no significant differences between feed additives. This shows that bacteriophage is a better feed additive compared with its counterparts; moreover, piglets supplemented with BA also showed improved performance compared to the control group. The positive effects of BA in the current study are in line with previous studies where supplementation with BA in single (Gebru et al. 2010) or cocktail (Kim et al. 2014) form improved ADG in pigs. An increase in growth rate could be attributed to the reduction of pathogenic bacteria such as *E. coli* and *Clostridium* spp. by BA in the intestine, which breaks down protein as an energy source for its metabolism (Macfarlane and Macfarlane 2003). ZnO and OA did not improve ADG; however, these antimicrobial agents are known to increase the growth of piglets during the early growing period (Waern et al. 1998; Suryanarayana et al. 2012).

In the present study, the ATTD of DM was increased only at the end of the experiment in all supplemented groups. Similarly, the ATTD of CP was also enhanced during Phase II for BA. This is in line with the earlier studies of Kim et al. (2014) who reported improved DM and CP digestibility in growing pigs. This improvement in digestibility in BA-supplemented diets might be associated with the significant improvements in the ADG and gut microflora of weaning pigs. The increase in the digestibility of DM in response to OA treatment might be due to the effects of these substances on digestible enzymes (Botermans et al. 1999) as they have the ability to affect pancreatic and bile secretion by diffusing into cells (Thaela et al. 1998). The increase in the digestibility in response to ZnO treatment might be related to improvements in gut morphology, i.e. increased villous height, which was also evident in the present study.

The evaluation of villus height and crypt depth in the different portions of the intestine, i.e. duodenum, jejunum and ileum, was done to assess the gut health of the pigs. Villi are the finger-like projections in the epithelial lining of the small intestine that help to increase surface area for digestion and absorption processes, while crypts are the tubular glands in the mucosal surface of the small intestine that open into lumen at the base of the villi and contain epithelial stem cells required for repopulation of epithelial cells (Llyod and Gabe 2008). It is evident from the literature that weaning causes tremendous structural changes to the pig intestine (Hampson 1986). In the present study, the villus height of the duodenum was significantly increased in the BA and ZnO groups. The higher villus height may have enhanced the surface area for absorption, in turn leading to increased nutrient digestion capacity. Furthermore, determination of intestinal microflora plays an important role in revealing gut health. In the present study, there was a decrease in the concentration of pathogenic bacteria such as *Clostridium* spp. and coliforms. This correlates with the earlier studies of Mourao et al. (2006) who reported that decreasing the number of pathogenic bacteria in the gut may improve the capacity epithelial cells to build villi and thus enhance intestinal morphology, influence intestinal morphology, either by neuroendocrine factors or by the products that these microorganisms secrete (Barbara et al. 2005). Lactic acid bacteria influence the distribution and the numbers of lymphoid cells in lymphatic tissues associated with the gut, ensure the balance in the composition of the gut microflora, and through their activity are able to maintain the integrity of the gut mucous membrane (Herich and Levkut 2002). Crypt depth did not significantly differ among the groups; however, *Bifidiobacteium* spp. and *Lactobacilli* spp. reduce the pH of the intestinal content by stimulating the production of lactic acid and by decreasing crypt depth (Salminen and Salminen 1997). In this study, we showed the beneficial effects of a BA cocktail on performance, gut development and microflora in weanling pigs. Thus, we conclude that the BA cocktail can be a suitable alternative to ZnO.

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