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Effects of dietary supplementation of antimicrobial peptide-A3 on growth performance, nutrient digestibility, intestinal and fecal microflora and intestinal morphology in weanling pigs

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ABSTRACT

In this study, effects of dietary supplementation of the antimicrobial peptide-A3 (AMP-A3) on growth performance, coefficient of total tract apparent digestibility (CTTAD) of nutrients, serum immunoglobulins, intestinal and fecal microflora and intestinal morphology in weanling piglets were evaluated when used as a substitute to the antibiotics. A total of 240 weanling piglets (Landrace × Yorkshire × Duroc, initial body weight (BW): 5.74 ± 0.38 kg) were randomly allotted to 4 treatments on the basis of BW. There were 4 replicate pens in each treatment with 15 pigs per pen. The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diets). The experimental diets were fed in a meal form for 2 phases (d 0–14, phase I and d 15–28, phase II post-weaning). Pigs fed the PC diet had greater (P<0.05) overall average daily gain (ADG), average daily feed intake (ADFI) and the CTTAD of crude protein (CP, phase I) and dry matter (DM) and CP (phase II) than pigs fed the AMP-A3 diet. Increasing levels of dietary AMP-A3 linearly improved (P<0.05) overall ADG and CTTAD of DM and CP (phase I and II). Pigs fed the PC diet had lower (P<0.05) coliforms (cecum and feces) and total anaerobic bacteria (TAB, cecum) than pigs fed the AMP-A3 diet. When pigs offered increasing levels of the AMP-A3 diets, there was a linear decline (P<0.05) in TAB, coliforms and *Clostridium* spp. in the ileum, cecum and feces (d 14 and 28). Pigs fed the PC diet had lower (P<0.05) crypt depth and greater (P<0.05) villus height to crypt depth ratio (VH:CD) of the jejunum than pigs fed the AMP-A3 diets. Increasing levels of AMP-A3 in the diets of pigs increased (linear, P<0.05) villus height and VH:CD and decreased (linear, P<0.05) crypt depth of the duodenum and jejunum. Dietary treatments had no effect (P>0.05) on the coefficient of ileal apparent digestibility of amino acids and serum immunoglobulin concentrations. The results obtained in the present study indicate that the AMP-A3 had beneficial effects on growth performance, CTTAD of nutrients, intestinal morphology and intestinal and fecal microflora and can be used as a potential alternative to antibiotic growth promoters in weanling pigs.

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; AMP-A3, antimicrobial peptide-A3; ATTD, apparent total tract digestibility; BW, body weight; CP, crude protein; DM, dry matter; G:F, gain:feed; GE, gross energy; NC, negative control; PC, positive control; TAB, total anaerobic bacteria; VH:CD, villus height:crypt depth ratio.

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1. Introduction

Many changes associated with weaning expose young piglets to a number of stressors that can lead to depressed feed intake, growth performance and increased disease and mortality (Okai et al., 1976; Pluske et al., 1996). This has led to the development of feed additives with high efficiency and low toxicity in order to boost host defense system of weanling pigs. Antibiotics are generally added to the weanling piglet diets to maintain the health and improve growth performance. Nevertheless, their continuous use and misuse has led to the emergence of drug resistance (Monroe and Polk, 2000) and risk of the antibiotic-residues in animal products (Schwarz et al., 2001). Therefore, the search continues for new antimicrobials that are active *in vivo*, are fast acting and broad-spectrum, do not include bacterial resistance and have limited side effects. In this sense, synthetic congeners of the natural antimicrobial peptides (AMP) are believed to be one of the ideal candidates, due to their natural antimicrobial properties, broad spectrum activity, speed of action and a low propensity for the development of bacterial resistance (Hancock and Lehrer, 1998; Bradshaw, 2003). It has speculated that unlike currently used antibiotics, acquisition of resistance by microbes against AMP is thought to be improbable, as AMP have numerous targets and making elimination of one target is less significant (Marr et al., 2006).

The AMP is small gene-encoded peptides that show a broad range of activity against gram-negative and gram-positive bacteria, fungi, and mycobacteria (Zasloff, 2002). The AMP acts against target organism either by membrane depolarisation, micelles formation or diffusion of AMP onto intracellular targets (Matsuzaki, 1999; Shai, 1999; Huang, 2000; Keymanesh et al., 2009). The interest of AMP as potential antibiotic pharmaceuticals has always been high. Because of their rapid and broad spectrum properties, these peptides were quickly proposed as antimicrobials to treat microbial infections, particularly those caused by antibiotic resistant bacteria (Hadley and Hancock, 2010). However, the use of AMP as feed additive is still in the stage of infancy with most of the work being conducted *in vitro* and very few animal studies have been documented. Positive effect of supplementation of various AMP on growth performance (Shan et al., 2007; Wang et al., 2011), nutrient digestibility (Jin et al., 2008a,b), intestinal microflora (Wang et al., 2007; Jin et al., 2009), intestinal morphology (Wang et al., 2006, 2007) and immune functions (Shan et al., 2007; Tang et al., 2009) has been reported previously. The objectives of the present experiment were to evaluate the effects dietary supplementation of synthetic antimicrobial peptide-A3 (AMP-A3) on growth performance, coefficient of total tract apparent digestibility (CTTAD) of nutrients, serum immunoglobulins, intestinal and fecal microflora and intestinal morphology in weanling pigs when it was used as an alternative to antibiotics.

2. Materials and methods

The protocol for the present experiment was approved by the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Republic of Korea. The experiment was conducted at the facility of Kangwon National University farm and the pigs (Landrace × Yorkshire × Duroc) were housed in partially slatted concrete floor pens with pen size 1.90 m × 2.54 m. All pens were equipped with a self-feeder and nipple drinker to allow *ad libitum* access to feed and water.

2.1. Peptide synthesis

The antimicrobial peptide (AMP-A3) used in the present study was provided by Research Center for Proteineous Materials, Chosun University, Kwangju, South Korea. The AMP-A3 (amino acid sequence: AKKVFKRLEKLFSKIWNWK-NH_2) is an analog of antimicrobial peptide HP 2-20 (amino acid sequence: AKKVFKRLEKLFSKIQNDK-NH_2) designed by substitution of amino acid tryptophan for the hydrophobic amino acids, glutamine and aspartic acid (Lee et al., 2002). In short, the AMP-A3 was synthesised by solid phase method using 9-fluorenyl-methoxycarbonyl (Fmoc) chemistry (Merrifield, 1986). Rink amide 4-methyl benzhydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a C-terminal amidate peptide. The coupling of Fmoc-L-amino acids was performed with N-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: tert-butyl (aspartic acid), trityl (glutamine), tert-butyloxycarbonyl (lysine). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol and triisopropylsilane (88:2.5:2.5:2.5:2.5:2.0, v/v) for 2 h at room temperature. The crude peptide was then repeatedly washed with diethylether, dried in vacuum, and purified using a reversed phase preparative HPLC on a Waters 15 Am Deltapak C18 column (19 cm × 30 cm). Purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere C18 column (Beckman, Fullerton, CA, USA), 4.6 cm × 25 cm.

2.2. Animals and experimental design

A total of 240 weanling piglets (Landrace × Yorkshire × Duroc; average initial body weight (BW): 5.74 ± 0.38 kg) were randomly allotted to 4 treatments on the basis of BW. There were 4 replicate pens in each treatment with 15 pigs per pen. The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet). The antibiotic used in present study was 0.15% apramycin (Apralan; KBNP Inc., Gunpocity, Kyungki-Do, Korea). The levels of the AMP-A3 used in present study were based upon the results of minimum inhibitory concentration (Lee et al., 2002). The experimental diets were fed in 2 phases: phase I (d 0–14 post-weaning) and phase II (d 15–28 post-weaning). Diets were formulated to contain 14.28 MJ/kg ME and 15.5 g/kg lysine (phase I) and 14.11 MJ/kg

Table 1
Ingredient and chemical composition of basal diets (as-fed basis).[a,b]

Item	Phase I (d 0–14 post-weaning)	Phase II (d 15–28 post-weaning)
Ingredients (g/kg)		
Maize	–	340.8
Maize (expanded)	222.4	140.0
Maize starch	80.0	–
Soybean meal (440 g crude protein/kg)	–	191.0
Soybean meal (d475 g crude protein/kg)	121.1	150.0
Whey powder	150.0	70.0
Soy protein concentrate	80.0	–
Fish meal	20.0	20.0
Animal fat	–	40.0
Lactose	110.0	–
Sucrose	30.0	10.0
Whey protein concentrate	60.0	–
Spray dried porcine plasma	45.0	–
Soy oil	45.0	–
Monocalcium phosphate	9.6	–
Dicalcium phosphate	–	11.5
Limestone	8.3	7.8
Zinc oxide	3.0	3.0
Vitamin premix[c]	2.5	2.5
Mineral premix[d]	1.5	1.5
Salt	2.0	2.5
Acidifier[e]	2.0	1.5
L-Lysine HCL (780 g/kg)	3.8	4.4
DL-Methionine (980 g/kg)	1.5	1.3
L-Threonine (980 g/kg)	1.3	1.2
Choline chloride (250 g/kg)	1.0	1.0
Chemical composition, calculated		
Metabolic energy (MJ/kg)	14.28	14.11
Crude protein (g/kg)	228.4	217.0
Lysine (g/kg)	15.5	13.5
Calcium (g/kg)	8.0	7.7
Available phosphorus (g/kg)	4.8	3.6

[a] The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet).
[b] For each of the AMP-A3 containing experimental treatments, the AMP-A3 was mixed with carrier (basal diet) in such way that addition of 0, 6.0 and 9.0 g/kg of AMP-A3 with carrier, would give 0, 60 and 90 mg/kg diet AMP-A3, respectively.
[c] Supplied per kg diet: 9600 IU vitamin A, 1800 IU vitamin D_3, 24 mg vitamin E, 1.5 mg vitamin B_1, 12 mg vitamin B_2, 2.4 mg vitamin B_6, 0.045 mg vitamin B_{12}, 1.5 mg vitamin K_3, 24 mg pantothenic acid, 45 mg niacin, 0.09 mg biotin, 0.75 mg folic acid, 18 mg ethoxyquin.
[d] Supplied per kg diet: 162 mg Fe (ferrous sulfate), 96 mg Cu (copper sulfate), 46.49 mg Mn (manganese sulfate), 0.9 mg I (calcium iodate), 0.9 mg Co (cobalt sulfate), 0.3 mg Se (sodium selenite).
[e] Complex of lactic acid, formic acid and phosphoric acid (1:1:1).

ME and 13.5 g/kg lysine (phase II; Table 1). All diets met or exceeded the nutrient requirements as suggested by NRC (1998; Table 2). The AMP-A3 was added to basal diets by using a portion of the basal diets as carrier. For each of the AMP-A3 containing experimental treatments, the AMP-A3 was mixed with carrier (basal diet) in such way that addition of 0, 6.0 and 9.0 g/kg of AMP-A3 with carrier, would give 0, 60 and 90 mg/kg diet AMP-A3, respectively.

2.3. Sample preparation and measurements

The pigs were individually weighed at the start of the trial and on d 14 and 28. Feed consumption was recorded at the end of each phase and the average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) were calculated. For the digestibility trial, diets containing 2.5 g chromium as indigestible marker/kg diet were fed to pigs during last seven days of each phase and then fecal grab samples were collected randomly from four pigs of each pen during last three days of each phase. About 100 g fecal sample was collected from each pig, feces were pooled and dried in an air forced drying oven at 60 °C for 72 h and ground in a Wiley laboratory mill (Thomas Model 4 Wiley® Mill, Thomas scientific, Swedesboro, NJ, USA) using a 1-mm screen and used for chemical analysis. Fresh fecal samples were collected from 2 pigs in each pen at d 14 and 28 and used for measuring fecal bacterial counts. The samples collected for microbial analysis were immediately placed on ice until the analyses was conducted later on the corresponding day. On the d 14 and 28 of experiment, a 10 ml blood sample was collected by jugular vein puncture from 2 randomly selected pigs in each pen using a disposable vacutainer tube without anticoagulants (Becton Dickinson, Franklin, NJ, USA). After centrifugation (3000 × *g* for 15 min at 4 °C), serum samples were separated and stored at −20 °C and later analysed for concentrations of immunoglobulins (IgG, IgA and IgM).

To study the effect of diets on coefficient of ileal apparent digestibility of amino acids, small intestinal morphology and microflora of large intestinal chyme, representative pigs from each treatment (2 per replicate) reflecting average body

Table 2
Analysed chemical composition of diets fed during digestibility study.[a,b]

Item	AMP-A3			
	0	60	90	PC
Phase I (8–14 d post-weaning)				
Dry matter (g/kg)	924.4	927.3	925.6	923.8
Crude protein (g/kg)	220.6	219.8	222.7	221.9
Ash (g/kg)	53.8	51.7	54.3	52.9
Calcium (g/kg)	8.7	8.2	8.5	8.4
Total P (g/kg)	5.5	5.7	5.8	5.4
Chromium (g/kg)	2.3	2.2	2.2	2.3
Phase II (22–28 d post-weaning)				
Dry matter (g/kg)	927.3	931.4	926.5	928.7
Crude protein (g/kg)	208.7	206.2	205.5	204.9
Ash (g/kg)	54.3	54.5	53.9	54.8
Calcium (g/kg)	9.0	9.3	8.9	9.0
Total P (g/kg)	4.9	5.1	4.9	5.0
Chromium (g/kg)	2.2	2.3	2.3	2.2
Essential amino acids (Phase II)				
Arginine	11.9	12.1	12.2	11.3
Histidine	4.9	5.0	5.0	4.9
Isoleucine	8.5	8.5	8.5	8.4
Leucine	18.7	19.0	19.0	18.9
Lysine	13.4	13.4	13.5	14.2
Metheonine	3.9	3.8	3.8	4.0
Phenylalanine	9.6	9.7	9.8	9.6
Threonine	9.3	9.5	9.5	9.4
Valine	9.8	10.0	10.0	9.8

[a] The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet).
[b] For each of the AMP-A3 containing experimental treatments, the AMP-A3 was mixed with carrier (basal diet) in such way that addition of 0, 6.0 and 9.0 g/kg of AMP-A3 with carrier, would give 0, 60 and 90 mg/kg diet AMP-A3, respectively.

weight were selected and sacrificed by electrocution at the end of the experiment (d 28). The chyme from the terminal ileum (about 20 cm from the ileo-ceacal junction) was collected in a sterilised plastic bottle, kept in an icebox and then brought to laboratory and freeze-dried until analysis of amino acids. The cecum contents were also collected in sterilised plastic bottle and stored at 7 °C for bacterial analysis later on the same day. The samples of intestinal segment from the region of the duodenum, jejunum and ileum after removal of its contents were flushed with physiological saline and submerged in a fixative solution (0.1 M collidine buffer, pH 7.3) containing 30 g/l glutaraldehyde, 20 g/l paraformaldehyde and 15 g/l acrolein and then brought to laboratory to study morphological changes.

2.4. Chemical and microbial analyses

Experimental diets and excreta samples were analysed in triplicate for dry matter (DM, method 930.15; AOAC, 2007), crude protein (CP, method 990.03; AOAC, 2007), ash (method 942.05; AOAC, 2007), calcium, and phosphorus (method 985.01; AOAC, 2007). The gross energy (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). Amino acid composition of feed samples and ileum contents were determined by HPLC (Waters 486, Waters Corp., Milford, MA, USA) after acid hydrolysis (Knabe et al., 1989). The methionine and cystine were determined following oxidation with performic acid (Moore, 1963). The concentrations of serum IgG, IgA and IgM were analysed using radial immune-diffusion kits (Tripple J Farms, Bellingham, WA, USA).

The microbiological assay of fecal samples and intestinal chyme was carried out by the procedure suggested by Torrallardona et al. (2003). One gram of mixed content was diluted with 9 ml of Butterfields phosphate buffer solution, followed by further serial dilutions in buffer-fields phosphate buffer dilution solution. Duplicate plates were then inoculated with 0.1 ml sample and incubated. The microbial groups enumerated were total anaerobic bacteria (TAB, plate count agar, Difco Laboratories, Detroit, MI, USA), coliforms (violet red bile agar, Difco Laboratories, Detroit, MI, USA) and *Clostridium* spp. (Tryptose sulphite cycloserine agar, Oxoid, Hampshire, UK). The anaerobic conditions during the assay of total anaerobic bacteria and *Clostridium* spp. were created by using gas-pak anaerobic system (BBL, No. 260678, Difco, Detroit, MI, USA). The microbial populations were log transformed before statistical analysis.

Table 3
Effect of the antimicrobial peptide-A3 (AMP-A3) supplementation on the growth performance of weanling pigs.[a]

Item	PC	AMP-A3			SEM[b]	P-value[c]		
		0	60	90		PC *vs.* AMP-A3	Linear	Quadratic
Phase I (d 0–14)								
Average daily gain (g)	242	220	225	232	5.70	0.018	0.016	0.922
Average daily feed intake (g)	360	337	344	349	5.93	0.031	0.145	0.872
Gain:Feed (g/kg)	673	651	655	665	5.37	0.398	0.314	0.781
Phase II (d 15–28)								
Average daily gain (g)	374	342	345	358	6.08	0.071	0.020	0.560
Average daily feed intake (g)	579	556	561	568	5.26	0.032	0.482	0.702
Gain:feed (g/kg)	645	616	615	630	5.46	0.096	0.400	0.599
Overall (d 0–28)								
Initial body weight (kg)	5.78	5.76	5.78	5.74	0.03	0.822	0.356	0.588
Final body weight (kg)	14.41	13.62	13.77	13.99	0.34	0.010	0.014	0.388
Average daily gain (g)	308	281	286	295	5.86	0.010	0.030	0.412
Average daily feed intake (g)	470	447	453	458	6.56	0.040	0.082	0.784
Gain:feed (g/kg)	659	633	635	664	4.91	0.048	0.170	0.529

[a] The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet).
[b] Standard error of the mean.
[c] Comparison between positive control *vs.* antimicrobial peptide-A3 (60 and 90 mg AMP-A3/kg diet). Linear and quadratic effects of increasing AMP-A3 concentration (0, 60 and 90 mg AMP-A3/kg diet).

2.5. Small intestinal morphology

Three cross-sections for each intestinal sample 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. Villus height was measured from the tip of the villi to the villus crypt junction. Crypt depth was defined as the depth of the invagination between adjacent villi. All morphological measurements (villus height or 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, MA, USA).

2.6. Statistical analyses

The data generated were analysed as a randomised complete block design using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The linear and quadratic contrasts were used to compare effect of increasing dietary AMP-A3 levels (0, 60 and 90 mg AMP-A3/kg). An independent-sample *t*-test was used to analyse the difference between PC and AMP-A3 (60 and 90 mg AMP-A3/kg). The pen was used as the experimental unit for the analysis of all the parameters. Probability values of $\leq$0.05 were considered as significant.

3. Results

3.1. Growth performance

Pigs fed the PC diet had greater (P<0.05; Table 3) final body weight, ADG (phase I and overall), ADFI (phase I, phase II and overall) and G:F (overall) than pigs fed the AMP-A3 diets. Dietary supplementation of increasing level of the AMP-A3 improved (linear, P<0.05) final body weight and ADG (phase I, phase II and overall period). However, dietary supplementation of increasing level of the AMP-A3 had no influence (linear or quadratic, P>0.05) on ADFI and G:F of pigs during phase I, phase II or overall study period.

3.2. Coefficient of digestibility

Pigs fed the PC diet had greater CTTAD of DM (d 14 and 28) and CP (d 14) than pigs fed the AMP-A3 diets. Pigs fed increasing levels of AMP-A3 had improved (linear, P<0.05; Table 4) CTTAD of DM and CP (d 14 and 28). However, dietary treatments had no effect (P>0.05) on coefficient of ileal apparent digestibility of essential and non-essential amino acids.

3.3. Fecal and intestinal microbial population

Pigs fed the PC diet had lower (P<0.05; Table 5) cecal TAB and coliform populations than pigs fed the AMP-A3 diets. The ileal TAB, *Clostridium* spp. and coliform populations of pigs fed PC diet were not significantly different from pigs fed the AMP-A3 diets. Increasing the level of the AMP-A3 in the diets of pigs linearly reduced (P<0.05) the populations of TAB, coliforms (ileum and cecum) and clostridia (cecum) on d 28.

Table 4
Effects of antimicrobial peptide-A3 (AMP-A3) supplementation on coefficient of total tract apparent digestibility of nutrients and coefficient of ileal apparent digestibility of amino acids in weanling pigs.[a]

Item	PC	AMP-A3			SEM[b]	P-value[c]		
		0	60	90		PC *vs.* AMP-A3	Linear	Quadratic
Phase I (d 0–14)								
Dry matter	0.82	0.79	0.80	0.82	0.03	0.028	0.001	0.881
Crude protein	0.81	0.77	79	0.80	0.03	0.020	0.010	0.121
Gross energy	0.83	0.80	0.82	0.82	0.04	0.466	0.251	0.586
Phase II (d 15–28)								
Dry matter	0.81	0.77	0.79	0.80	0.04	0.040	0.020	0.351
Crude protein	0.80	0.75	0.78	0.79	0.05	0.277	0.001	0.180
Gross energy	0.81	0.77	0.79	0.80	0.07	0.301	0.084	0.737
Essential amino acids								
Arginine	0.80	0.79	0.79	0.79	0.06	0.689	0.762	0.977
Histidine	0.72	0.71	0.71	0.71	0.07	0.777	0.994	0.923
Isoleucine	0.71	0.70	0.70	0.70	0.06	0.324	0.949	0.962
Leucine	0.74	0.73	0.73	0.73	0.05	0.545	0.914	0.990
Lysine	0.69	0.67	0.68	0.69	0.07	0.721	0.649	0.934
Metheonine	0.81	0.80	0.79	0.80	0.03	0.170	0.586	0.644
Phenylalanine	0.74	0.73	0.73	0.74	0.05	0.947	0.470	0.802
Threonine	0.65	0.64	0.64	0.65	0.05	0.519	0.951	0.937
Valine	0.65	0.64	0.65	0.65	0.07	0.657	0.543	0.547
Non-essential amino acids								
Alanine	0.65	0.63	0.64	0.63	0.06	0.886	0.826	0.998
Aspartic acid	0.71	0.70	0.70	0.71	0.05	0.920	0.915	0.893
Cystine	0.58	0.55	0.56	0.55	0.07	0.906	0.744	0.945
Glutamic acid	0.74	0.73	0.74	0.74	0.04	0.664	0.952	0.904
Glycine	0.49	0.48	0.50	0.49	0.08	0.612	0.583	0.900
Serine	0.72	0.70	0.71	0.71	0.06	0.942	0.931	0.990
Tyrosine	0.74	0.73	0.72	0.73	0.04	0.904	0.913	0.970

[a] The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet).
[b] Standard error of the mean.
[c] Comparison between positive control *vs.* antimicrobial peptide-A3 (60 and 90 mg AMP-A3/kg diet). Linear and quadratic effects of increasing AMP-A3 concentration (0, 60 and 90 mg AMP-A3/kg diet).

Table 5
Effects of antimicrobial peptide-A3 (AMP-A3) on bacterial populations (log_{10} CFU/g) in feces, ileum and cecum of weanling pigs (d 28).[a]

Item	PC	AMP-A3			SEM[b]	P-value[c]		
		0	60	90		PC *vs.* AMP-A3	Linear	Quadratic
Ileum (d 28)								
Total anaerobic bacteria	8.23	8.63	8.43	8.29	0.05	0.133	0.007	0.736
Clostridium spp.	7.15	7.36	7.28	7.16	0.04	0.419	0.093	0.805
coliforms	5.97	6.42	6.22	6.09	0.05	0.076	0.005	0.627
Cecum (d 28)								
Total anaerobic bacteria	8.26	8.71	8.49	8.35	0.05	0.043	0.001	0.525
Clostridium spp.	7.19	7.50	7.33	7.24	0.04	0.202	0.011	0.651
coliforms	5.98	6.43	6.24	6.12	0.05	0.007	0.001	0.493
Feces (d 14)								
Total anaerobic bacteria	8.42	9.37	8.70	8.63	0.11	0.144	0.005	0.127
Clostridium spp.	7.39	8.06	7.49	7.42	0.09	0.188	0.008	0.158
Coliforms	6.12	6.81	6.41	6.28	0.07	0.001	0.001	0.051
Feces (d 28)								
Total anaerobic bacteria	7.29	8.78	7.57	7.37	0.16	0.092	0.001	0.152
Clostridium spp.	7.06	7.51	7.21	7.12	0.05	0.110	0.010	0.144
Coliforms	5.74	6.53	6.07	5.97	0.08	0.010	0.001	0.165

[a] The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet).
[b] Standard error of the mean.
[c] Comparison between positive control *vs.* antimicrobial peptide-A3 (60 and 90 mg AMP-A3/kg diet). Linear and quadratic effects of increasing AMP-A3 concentration (0, 60 and 90 mg AMP-A3/kg diet).

Table 6
Effects of antimicrobial peptide-A3 (AMP-A3) on serum immunoglobulins (mg/ml) of weanling pigs.[a]

Item	NC	AMP-A3			SEM[b]	P-value[c]		
		0	60	90		PC *vs.* AMP-A3	Linear	Quadratic
d 14								
IgG	6.78	6.21	6.32	6.43	0.22	0.274	0.770	0.992
IgA	0.41	0.32	0.35	0.37	0.04	0.613	0.602	0.956
IgM	0.89	0.71	0.73	0.79	0.05	0.253	0.574	0.878
d 28								
IgG	6.87	6.31	6.39	6.53	0.21	0.493	0.527	0.926
IgA	0.43	0.36	0.39	0.42	0.09	0.746	0.632	0.886
IgM	0.92	0.73	0.79	0.81	0.04	0.281	0.507	0.820

[a] The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet).
[b] Standard error of the mean.
[c] Comparison between positive control *vs.* antimicrobial peptide-A3 (60 and 90 mg AMP-A3/kg diet). Linear and quadratic effects of increasing AMP-A3 concentration (0, 60 and 90 mg AMP-A3/kg diet).

Table 7
Effect of antimicrobial peptide-A3 (AMP-A3) supplementation on small intestinal morphology in weanling pigs (d 28).[a]

Item	PC	AMP-A3			SEM[b]	P-value[c]		
		0	60	90		PC *vs.* AMP-A3	Linear	Quadratic
Villus height (μm)								
Duodenum	547	495	533	553	8.47	0.819	0.004	0.527
Jejunum	601	544	576	595	8.39	0.417	0.023	0.680
Ileum	445	433	490	427	12.86	0.706	0.102	0.891
Crypt depth (μm)								
Duodenum	309	358	328	313	8.24	0.583	0.033	0.620
Jejunum	312	368	349	332	6.46	0.023	0.018	0.932
Ileum	248	288	252	231	10.33	0.782	0.075	0.777
Villus height to crypt depth ratio								
Duodenum	1.78	1.38	1.64	1.78	0.05	0.541	0.008	0.567
Jejunum	1.93	1.48	1.65	1.79	0.06	0.007	0.001	0.689
Ileum	1.84	1.52	1.98	1.91	0.10	0.679	0.168	0.279

[a] The dietary treatments were: PC (positive control; basal diet + 1.5 g apramycin/kg diet) and AMP-A3 (basal diet supplemented with 0, 60 and 90 mg AMP-A3/kg diet).
[b] Standard error of the mean.
[c] Comparison between positive control *vs.* antimicrobial peptide-A3 (60 and 90 mg AMP-A3/kg diet). Linear and quadratic effects of increasing AMP-A3 concentration (0, 60 and 90 mg AMP-A3/kg diet).

At d 14 and 28, pigs fed the PC diet had lower (P<0.05; Table 5) fecal coliforms population than pigs fed the AMP-A3 diets. Pigs fed diets supplemented with increasing level of the AMP-A3 had reduced (linear, P<0.05) fecal populations of TAB, *Clostridium* spp. and coliforms (d 14 and 28).

3.4. Serum immunoglobulins

Dietary treatments had no significant effect (P>0.05; Table 6) on serum immunoglobulins (IgG, IgA and IgM) concentration at d 14 and 28.

3.5. Intestinal morphology

Pigs fed the PC diet had reduced (P<0.05; Table 7) crypt depth and increased villus height to crypt depth ratio (VH:CD) of the jejunum than pigs fed the AMP-A3 diets. Increasing the level of AMP-A3 in the diets of pigs linearly increased (P<0.05) villus height and VH:CD and decreased (linear, P<0.05) crypt depth of the duodenum and jejunum. However, dietary treatment had no effect (P>0.05) on morphology of the ileum.

4. Discussion

In recent years, considerable efforts have been made for developing novel alternative to antibiotic feed additives. Among these alternatives, the antimicrobial peptides (AMP's) have received attention due to its broad spectrum activity, speed of action and a low propensity for the development of bacterial resistance (Hancock and Lehrer, 1998; Bradshaw, 2003; Wang et al., 2006; Jin et al., 2008a,b). The present study demonstrates the effect of the AMP-A3 as potential feed additive in weanling piglets nutrition. The antibiotic, apramycin was used with the objective of evaluating the potential of the AMP-A3 as an alternate to antibiotic growth promoters.

The improved growth performance in weanling pigs fed diets supplemented with increasing levels of the AMP-A3 that was observed in present study is in good agreement with data reported by Jin et al. (2008a,b) who observed improvement in ADG and feed efficiency of weanling pigs fed diets supplemented with increasing levels antimicrobial peptide from *Solanum tuberosum*. Similarly, Wen et al. (2001) also observed improvement in ADG of weanling pigs fed diets supplemented with the antimicrobial peptides. In contrast to the report of Wen et al. (2001) and the present results, Shan et al. (2007) reported no effects of dietary supplementation of antimicrobial peptide (lactoferrin) on growth performance of weanling pigs. This variation in results might be due to variation in type of the antimicrobial peptides used, level of dietary supplementation or mode of action of the antimicrobial peptides. In this experiment, as like in others (Wang et al., 2006; Jin et al., 2009; Tang et al., 2009) weanling pigs fed diets supplemented with antibiotic had greater overall ADG. The greater ADG in pigs fed the antibiotic diets than pigs fed the AMP-A3 diets in present study might be associated with greater feed intake and improved feed efficiency in pigs fed the antibiotic diet, as is also evident by greater DM and CP digestibility, reduction in *Clostridium* and coliforms populations in pigs fed diet supplemented with the antibiotic.

In the present study, supplementation of antibiotics and increasing levels of the AMP-A3 to weanling pigs diets had greater CTTAD of DM and CP. Result obtained in present study are consistent with findings of Jin et al. (2008a,b) who reported an increase in CTTAD of DM and CP in weanling pigs fed diet supplemented with increasing level of potato antimicrobial peptide. Similar to present results, improved digestibility of nutrients in weanling pigs fed the diet supplemented with the apramycin were reported in previous studies (Hu et al., 2008; Jin et al., 2008a,b; Choi et al., 2011). Improved apparent total tract digestibility of nutrients with the antibiotics might be due to increased availability of nutrients for intestinal absorption and by suppressing growth and metabolic activities of harmful gut microflora with simultaneous alteration in intestinal morphology, intestinal epithelium thickness and epithelial cell turnover (Jin et al., 2008a,b; Choi et al., 2011). On the other hand, apparent total tract digestibility of nutrients in pigs fed diets supplemented with the antimicrobial peptides might be due to modulation of gut environment, improvement of intestine microbial balance and stimulation of mucosal immune system (Wang et al., 2007; Jin et al., 2008a,b; Tang et al., 2009). In this experiment dietary supplementation of the antibiotics and AMP-A3 to weanling pigs diets had no effects on the coefficient of ileal apparent digestibility of the essential and nonessential amino acids. Present findings are consistent with Jin et al. (2009) who also reported that piglets fed diet supplemented with potato antimicrobial peptide have no effects on ileal apparent digestibility essential and non-essential amino acids.

The weaning piglets own immune system begins developing, but is not able to mount an effective immune response until the pigs is about 4–5 wk of age (Wang et al., 2004). The newly weaned piglets are very susceptible to diseases and stressors during first 2 wk postweaning. At this stage, nonspecific immunity factors are more important to the growth performance and immunity of weanling piglets (Bosi et al., 2003). The antimicrobial peptides are important components of the nonspecific immune system and supplementation of antimicrobial peptide (lactoferrin) could improve the growth performance and immunity of piglets (Wang et al., 2006; Shan et al., 2007). However, in current study, supplementation of weanling pigs diets with the apramycin or AMP-A3 had no effects on serum immunoglobulin concentrations. Contrary to present results, Shan et al. (2007) reported an increase in serum IgG, IgA, (d 15 and 30) and IgM (d 15) concentration in weanling pigs fed diet supplemented with antimicrobial peptide (lactoferrin). Tang et al., 2009 also observed increased serum IgG, IgA and IgM in weanling piglets fed diet supplemented with an expressed fusion peptide bovine lactoferricin–lactoferrampin. The increased concentration of the serum immunoglobulin are required to regulate and enhance the immune functions, which provides health benefits, diminish weaning stress and improve health status and growth performance of weanling pigs (Turner et al., 2002). Our results indicate that improved growth performance in present study is not due to enhanced immune function but might be due to improved gut barrier function *via* exclusion of pathogenic microorganisms (*Clostridium* and coliforms) and improved intestinal morphology.

The antimicrobial peptides beneficially affect the host animals by improving its intestinal balance and creating gut micro-ecological conditions that suppress harmful microorganisms like *Clostridium* spp. and coliforms and by favouring beneficial microorganisms like *Lactobacillus* and *Bifidobacterium* (Wang et al., 2007; Jin et al., 2008a,b; Tang et al., 2009; Ohh et al., 2010). In the present experiment, dietary supplementation of increasing levels of the AMP-A3 had potential of reducing harmful microflora like the fecal and intestinal TAB, coliforms and *Clostridium* spp. in weanling pigs. Some of the previous studies with supplementation of the potato antibacterial peptides (Jin et al., 2008a,b; Ohh et al., 2010) or expressed fusion peptide bovine lactoferricin–lactoferrampin (Tang et al., 2009) reported potential of antimicrobial peptides to suppress pathogenic coliforms and *Clostridium spp.* count. The reduced coliforms and *Clostridium* spp. in feces and intestine in pigs fed diet supplemented with antibiotics that was observed in present study are in good agreement with Jin et al. (2009) who observed reduced TAB, coliforms and *Staphylococcus* spp. in feces and large intestine of weanling pigs fed diet supplemented with antibiotics. Our results suggest that the AMP-A3 has potential for suppressing harmful intestinal microflora in weanling pigs and can be used as a potential alternative to the antibiotics.

The intestinal morphology including villus height, crypt depth and VH:CD of the duodenum, jejunum and ileum is indicative of the gut health in pigs. Increasing the villus height suggest an increased surface area capable of greater absorption of available nutrients (Caspary, 1992). The villus crypt is considered as villus factory and deeper crypts enhance tissue turnover to permit renewal of the villus required in response to normal sloughing or inflammation from pathogen and high demand for tissue in growing animals (Yason et al., 1987). Increased villus height and VH:CD are directly correlated with an increased epithelial turnover (Fan et al., 1997), and longer villi are correlated with activation of cell mitosis (Samanya and Yamauchi, 2002). In the present study, dietary supplementation of increasing levels of the AMP-A3 reported increase in villus height

and VH:CD and decrease in crypt depth of the duodenum and jejunum. Similarly, it was reported that weanling pigs fed diet supplemented with antimicrobial peptides (lactoferricin and lactoferrampin) had increased villus height and villus height to crypt depth ratio of the jejunum and ileum (Tang et al., 2009). Wang et al. (2006) reported increase in villus height and decrease in crypt depth at the small intestinal mucosa of the pigs fed diets supplemented with the antimicrobial peptide, lactoferrin. In contrast, Jin et al. (2008a,b) reported that there were no effects of dietary supplementation of potato antimicrobial peptide on the intestinal morphology of weanling pigs. The histomorphological changes in the intestine of weanling piglets reported in the present study provide new information regarding the potential for using the AMP-A3 as an alternative to the antibiotic growth promoters in pigs.

5. Conclusion

The results obtained in present study indicate that the AMP-A3 had beneficial effects on the growth performance, CTTAD of DM and CP, intestinal morphology and intestinal and fecal microflora and can be used as a potential alternative to the antibiotic growth promoters in weanling pigs.

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