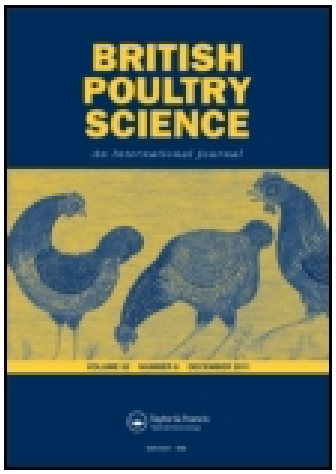

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An antimicrobial peptide-A3: effects on growth performance, nutrient retention, intestinal and faecal microflora and intestinal morphology of broilers

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Abstract 1. The present study investigated the effects of dietary supplementation with an antimicrobial peptide-A3 (AMP-A3) on growth performance, nutrient retention, intestinal microflora and intestinal morphology of broilers.

2. A total of 320-d-old chicks (Ross 308, average BW 44.0 ± 3.4 g) were randomly allotted to 4 dietary treatments on the basis of initial body weight (BW). The dietary treatments were negative control (NC; basal diet), positive control (PC; basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 or 90 mg/kg AMP-A3). The NC diet was considered as 0 mg/kg AMP-A3 treatment. Experimental diets were given in two phases: starter phase (d 0–21) and finisher phase (d 22–35).

3. The overall BW gain and retention of dry matter (DM), gross energy (GE; d 19–21) and crude protein (CP; d 19–21 and d 33–35) were greater in birds fed on the PC and 90 mg/kg AMP-A3 diets than in birds fed on the NC diet. Also, an increase in dietary AMP-A3 linearly improved BW gain and retention of DM, GE (d 19–21) and CP (d 19–21 and d 33–35).

4. Birds fed on the PC and 90 mg/kg AMP-A3 diets had fewer excreta coliforms (d 21 and d 35), total anaerobic bacteria (TAB) and *Clostridium* spp. (d 35) and ileum and caecum coliforms (d 35) than birds fed on the NC diet. In addition, birds fed on the diet supplemented with increasing levels of AMP-A3 had linearly reduced excreta TAB (d 35), *Clostridium* spp. and coliforms (d 21 and d 35) and ileum and caecum coliforms (d 35).

5. Birds fed on the PC and 90 mg/kg AMP-A3 diets had greater villus height of the duodenum, jejunum and ileum than birds fed on the NC diet. Moreover, birds fed on increasing levels of AMP-A3 diet had increased (linear) villus height of the duodenum, jejunum and ileum.

6. These results indicate that 90 mg/kg AMP-A3 has the potential to improve growth performance, nutrient retention and intestinal morphology and to reduce harmful microorganisms in broilers and can be used as a potential antimicrobial growth promoter.

INTRODUCTION

The emergence of bacterial strains exhibiting resistance against conventional antibiotics and the ban on the antibiotic growth promoters worldwide has urged the search for a novel means of preventing bacterial infection and promoting growth performance (Van den Bogaard and Stobberingh, 2000). Among the compounds that are presently under investigation, the antimicrobial peptides (AMPs) of the innate immune system and their synthetic derivatives are believed to be 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 been speculated that unlike currently used antibiotics, acquisition of resistance against AMPs is thought to be improbable, as AMPs have numerous targets, making elimination of one target less significant (Marr *et al.*, 2006).

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The AMPs are small gene-encoded peptides produced by wide variety of organisms as a part of non-specific immune response and are involved in direct destruction of microorganisms (Zasloff, 2002). The AMPs act against target organisms either by membrane depolarisation, micelle formation or diffusion of AMPs into intracellular targets (Matsuzaki, 1999; Shai, 1999; Huang, 2000; Keymanesh *et al.*, 2009). Positive effects of supplementation of various natural AMPs on growth performance (Wang *et al.*, 2009; Ohh *et al.*, 2010), intestinal morphology and mucosal immunity (Liu *et al.*, 2008; Bao *et al.*, 2009), excreta and caecal microbiology (Ohh *et al.*, 2009) of broilers have been documented. The mechanism of action of AMPs is based on the structural properties such as their amino acid sequences, size, cationic nature, hydrophobicity and amphipathicity that govern their interaction with target cells (Keymanesh *et al.*, 2009). It was reported that the antimicrobial activity of the natural AMPs can be improved by designing analogue peptides by modifying the structural properties of the natural peptides (Maloy and Kari, 1995; Javadpour *et al.*, 1996). A previous study in the author's lab reported that dietary supplementation of antimicrobial peptide-A3 (AMP-A3) (designed by substitution of amino acids) had beneficial effects on growth performance, nutrient digestibility and intestinal microflora and morphology of weanling pigs (Yoon *et al.*, 2012). Hence, the present study was undertaken with the objectives of determining the effects of dietary supplementation of AMP-A3 on growth performance, nutrient retention, excreta and intestinal microflora and intestinal morphology of broilers.

MATERIALS AND METHODS

The protocol for this experiment was approved and birds were cared for according to the guidelines of the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Republic of Korea.

Peptide synthesis

The antimicrobial peptide (AMP-A3) used in the present study was provided by Research Center for Proteinaceous Materials, Chosun University, Kwangju, Republic of Korea. The AMP-A3 (amino acid sequence: AKKVFKRLEKLFSKIWNWK–NH_2) is an analogue of antimicrobial peptide *Helicobacter pylori* 2–20 (HP 2–20; amino acid sequence: AKKVFKRLEKLFSKIQNDK–NH_2) designed by the 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-fluorenylmethoxycarbonyl (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 diethyl ether, dried in vacuum and purified using a reversed-phase preparative High performance liquid chromatography on a 15 Am Deltapak C18 column (19 × 30 cm (Waters Corporation, Milford, MA, USA)). Purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere C18 column (4.6 × 25 cm) (Beckman, Fullerton, CA, USA).

Birds, diets and management

A total of 320-d-old chicks (Ross 308, average BW 44.0 ± 3.4 g) were randomly allotted to 4 dietary treatments on the basis of body weight (BW). There were 4 replicate pens in each treatment with 20 chicks per pen. The dietary treatments were negative control (NC; basal diet without any antimicrobials), positive control (PC; basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 or 90 mg/kg AMP-A3). The NC (basal diet) was considered as 0 mg/kg AMP-A3. The levels of the AMP-A3 used in present study were based upon the minimum inhibitory concentration (Lee *et al.*, 2002) and results of dietary supplementation of AMP-A3 in weanling pigs (Yoon *et al.*, 2012). The basal diet was in mash form and was formulated for starter (d 0–21) and finisher (d 22–35) periods (Table 1). Avilamycin (Elanco, Liverpool, UK) and AMP-A3 were added to the basal diets at the expense of maize. All nutrients met or exceeded the nutrient requirements as recommended by NRC (1994).

The birds were housed in rice hull-covered floor pens. Each pen was provided with a self-feeder and hanging bell drinker to allow free access to feed and water. The house temperature was maintained at 34°C for the first 5 d and was then gradually reduced according to normal management practices, until a temperature of 23°C was achieved. Lighting was provided for 23 h/d.

Experimental procedures

The birds were individually weighed at the start of the trial and on d 21 and 35. Feed that was not consumed was weighed at end of each phase and

Table 1. *Ingredients and chemical composition of basal diets (as-fed basis)*[1]

Item	Starter (d 0–21)	Finisher (d 22–35)
Ingredient, g/kg		
Maize	556.8	583.4
Soybean meal (440 g crude protein/kg)	261.9	214.3
Wheat	20.0	50.0
Maize gluten meal	70.0	80.0
Fish meal (550 g crude protein/kg)	20.0	–
Animal fat	–	38.6
Soy-oil	36.5	–
Dicalcium phosphate	18.4	16.6
Limestone	7.5	7.5
Sodium chloride	3.0	3.0
L-Lysine (780 g/kg)	1.1	2.2
DL-Methionine (500 g/kg)	1.8	0.3
Choline chloride (500 g/kg)	1.0	1.3
Vitamin premix[2]	1.0	1.3
Trace mineral premix[3]	1.0	1.5
Chemical composition, calculated		
Metabolisable energy, MJ/kg	13.40	13.40
Crude protein, g/kg	218.9	196.7
Calcium, g/kg	9.3	9.7
Available phosphorus, g/kg	4.6	4.1
Lysine, g/kg	11.6	10.1
Methionine, g/kg	5.2	4.0
Methionine + Cysteine, g/kg	8.8	7.2

[1]The dietary treatments were the following: NC: negative control (basal diet without any antimicrobials); PC: positive control (basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 and 90 mg/kg AMP-A3). The AMP-P5 was added to diets at expense of maize.

[2]Provides (per kg diet): vitamin A palmitate, 9000 IU; cholecalciferol, 1800 IU; DL-α-tocopheryl acetate, 30 mg; menadione, 1 mg; thiamin, 1 mg; riboflavin, 10 mg; pyridoxine, 4 mg; cyanocobalamin, 0.02 mg; niacin, 30 mg; pantothenic acid, 12 mg; folic acid, 0.5 mg; biotin, 0.2 mg.

[3]Provides (per kg diet): 80 mg Fe (ferrous sulphate), 6 mg Cu (copper sulphate), 70 mg Zn (zinc sulfate), 84 mg Mn (manganese sulphate), 1.4 mg I (calcium iodate), 0.07 mg Co (cobalt sulphate), 0.2 mg Se (sodium selenite).

feed intake was calculated for starter (d 0–21), finisher (d 21–35) and for the overall study period (d 0–35). Body weight (BW) gain and feed intake were calculated by dividing total pen weight gain and total pen feed consumption by the number of birds d (including BW gain and feed intake of all dead birds in the pen). The feed conversion ratio (FCR) for each pen was calculated by dividing the feed intake by the BW gain. Two nutrient retention trials were conducted during the last week of each phase to determine retention of dry matter (DM), crude protein (CP) and gross energy (GE). From d 14 (starter) and d 28 (finisher) onwards, two birds from each replicate were allocated in individual cages (one bird/cage) to facilitate the collection of excreta samples. The starter and finisher diets containing 2.5 g/kg chromium as an indigestible marker were given from d 15 and 28 onwards, respectively. Excreta samples (about 100 g/d per bird) were collected from each bird for the last 3 d of each phase. The excreta samples collected for 3 d were pooled and dried in a forced air 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 were used for chemical analysis. Additionally, fresh excreta samples were collected from each bird housed in individual cages on d 21 and 35 and were used for measuring faecal bacterial counts. The samples collected for microbial analysis were immediately placed on ice (2–3 h) and were transported to the laboratory for further analysis on the same day. During the morning hours (08:00 h) of these corresponding days, the plastic liners placed in the excreta collection trays underneath each cage were replaced, and fresh clean excreta (free from feathers and feed) was collected.

To study the intestinal morphology and microflora of ileum and caecum contents, representative birds from each treatment (two per replicate) reflecting average BW were selected and slaughtered at the end of the experiment (d 35). The samples of fresh excreta, ileal and caecal contents were collected in sterile plastic bottles and were immediately placed on ice until the analysis was conducted later on the corresponding day. To study intestinal morphology, samples of intestinal segment were also collected from the region of duodenum, jejunum and ileum and 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 3% glutaraldehyde, 2% para-formaldehyde and 1.5% acrolein and were then brought to the laboratory to study the morphological changes.

Chemical and microbial analyses

Dry matter and CP analysis of experimental diets and excreta samples were done according to the Association of Official Analytical Chemists International (1995) methods (930.05 and 976.05, respectively). The GE of diets and faeces were measured by a bomb calorimeter (Model 1261, Parr Instrument Co., Moline, IL, USA) and chromium was measured with an atomic absorption spectrophotometer (Model AA-680G, Shimadzu, Kyoto, Japan) according to the procedure of Fenton and Fenton (1979). The excreta, ileal and caecal microflora were analysed according to the procedure of Jin *et al.* (2008). The microbial groups analysed were total anaerobic bacteria (TAB; plate count agar, Difco Laboratories, Detroit, MI), *Clostridium* spp. (Tryptose sulphite cycloserine agar, Oxoid, Hampshire, UK) and coliforms (violet red bile agar, Difco Laboratories). The anaerobic conditions during the assay of total anaerobic bacteria and *Clostridium* spp. were created by using a Gaspak anaerobic system (BBL, No. 260678, Difco, Detroit, MI, USA). The microbial counts were log transformed before statistical analysis.

Small intestine morphology

The small intestinal morphology was analysed according to the procedure of Yoon *et al.* (2012). In short, 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 villus 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 software version 6.5, Media Cybergenetics, North Reading, MA).

Statistical analysis

The data generated were analysed as a randomised complete block design by SAS (SAS Inst., Inc., Cary, NC). The one-way analysis of variance (ANOVA) test was used for analysis of all parameters, and when significant differences were determined among treatment means, they were separated by using Tukey's highly significant difference test. In addition, orthogonal polynomial contrasts were used to determine significant linear and quadratic response to increasing dietary AMP-A3 concentrations (0, 60 and 90 mg/kg). The NC (diet without antimicrobials) was considered as 0 mg/kg AMP-A3. A pen of birds was the experimental unit for all the analysis. Comparisons with $P \leq 0.05$ were considered significant.

RESULTS

Growth performance

Birds given the PC and 90 mg/kg AMP-A3 starter and finisher diets had greater ($P < 0.05$; Table 2) BW gain than birds given the NC and 60 mg/kg AMP-A3 diets. In addition, BW gain was linearly improved ($P < 0.05$) as the concentration of the AMP-A3 was increased in the starter and finisher diets. For the overall period, birds given the PC and 90 mg/kg AMP-A3 diets had greater ($P < 0.05$) BW gain than birds given the NC diet.

Table 2. *Effect of dietary supplementation of antimicrobial peptide-A3 (AMP-A3) on growth performance of broilers*[1,2]

		AMP-A3, mg/kg				*P*-values[3]		
Item	PC	0 (NC)	60	90	SEM	T	L	Q
Starter (d 0–21)								
Body weight gain, g	732[a]	693[b]	704[b]	722[a]	4.48	0.010	0.002	0.525
Feed intake, g	1143	1114	1123	1136	4.68	0.126	0.140	0.902
FCR[4]	1.56	1.61	1.60	1.57	0.01	0.080	0.119	0.680
Finisher (d 22–35)								
Body weight gain, g	1153[a]	1077[c]	1088[c]	1121[b]	10.21	0.016	0.043	0.543
Feed intake, g	2075	2013	2010	2048	15.63	0.439	0.326	0.500
FCR	1.80	1.87	1.85	1.83	0.01	0.098	0.085	0.904
Overall (d 0–35)								
Body weight gain, g	1885[a]	1769[c]	1792[bc]	1843[ab]	13.50	0.001	0.004	0.341
Feed intake, g	3218	3127	3134	3184	17.84	0.217	0.158	0.510
FCR	1.71[b]	1.77[a]	1.75[ab]	1.73[ab]	0.01	0.034	0.056	0.935

[a,b,c]Mean values within the same row sharing a common superscript letter are not statistically different at $P < 0.05$.

[1]The dietary treatments were the following: NC: negative control (basal diet without any antimicrobials); PC: positive control (basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 and 90 mg/kg AMP-A3). The NC (diet without antimicrobials) was considered as 0 mg/kg AMP-A3.

[2]Data are means of 4 pens of 20 birds each.

[3]T: overall effect of treatments; L: linear effect of increasing AMP-A3; Q: quadratic effect of increasing AMP-A3 (0, 60 and 90 mg/kg of diet).

[4]FCR, feed conversion ratio.

Also, birds given the PC diet had better ($P < 0.05$) overall FCR than birds given the NC diet. The overall BW gain and FCR of birds given the 90 mg/kg AMP-A3 diet were not different ($P < 0.05$) from the birds that were given the PC diet, whereas overall BW gain and FCR of birds given 60 mg/kg AMP-A3 diet were not different ($P < 0.05$) from birds given the NC and 90 mg/kg AMP-A3 diets. Moreover, the overall BW gain improved (linear, $P < 0.05$) with increasing AMP-A3 inclusion in the diets. Also, the overall FCR tended to improve ($P < 0.10$) as the concentration of AMP-A3 was increased in the diets. Dietary treatments had no effect on the feed intake of birds (starter, finisher and overall).

Nutrient retention

The retention of DM (d 19–21), GE (d 19–21) and CP (d 19–21 and d 33–35) was greater ($P < 0.05$; Table 3) in the birds given the PC and 90 mg/kg AMP-A3 diets than birds fed on the NC diet. The retention of DM, GE (d 19–21) and CP (d 33–35) in birds fed on the 90 mg/kg AMP-A3 diet was not different from birds fed on the PC diet, whereas the retention of DM, GE (d 19–21) and CP (d 33–35) in birds fed on the 60 mg/kg AMP-A3 diet was not different form birds fed on the NC and 90 mg/kg AMP-A3 diets. In addition, retention of DM, GE (d 19–21) and CP (d 33–35) was linearly increased ($P < 0.05$) with increasing AMP-A3 inclusion in the diet.

Excreta and intestinal microflora

Birds given the PC and 90 mg/kg AMP-A3 diets had fewer ($P < 0.05$; Table 4) excreta coliforms (d 21 and 35), TAB and *Clostridium* spp. (d 35) than birds given the NC diet. On d 21, birds given the PC diet had less ($P < 0.05$) excreta *Clostridium* spp. than birds given the NC diet. The excreta *Clostridium* spp., (d 21 and 35), TAB and coliforms (d 35) in birds given the 90 mg/kg AMP-A3 diet were not different ($P > 0.05$) from birds given the PC diet, whereas the excreta *Clostridium* spp., coliforms (d 21 and 35) and TAB (d 35) in birds given the 60 mg/kg AMP-A3 were not different form birds given the NC and 90 mg/kg AMP-A3 diets. In addition, increasing concentrations of AMP-A3 were effectively (linear, $P < 0.05$) reducing *Clostridium* spp. coliforms (d 21 and 35) and TAB (d 35) in the excreta.

On d 35, the coliforms (caecum and ileum) and TAB (caecum) populations were lowest ($P < 0.05$; Table 5) in birds given the PC diet, whereas it was highest ($P < 0.05$) in birds given the NC diet. Birds given the 90 mg/kg AMP-A3 diet had lesser ($P < 0.05$) coliforms in the ileum and caecum content than birds given the NC diet. The content of caecum TAB and coliforms of birds given the 90 mg/kg AMP-A3 diet did not differ from that of birds given the PC diet, whereas the caecum TAB and coliforms of birds given the 60 mg/kg AMP-A3 diet were not different from birds given the NC and 90 mg/kg AMP-A3 diets. Also, coliform populations in the caecum and ileum contents were linearly ($P < 0.05$) reduced as the dietary inclusion of AMP-A3 was increased.

Intestinal morphology

Birds fed on the PC and 90 mg/kg AMP-A3 diet had greater ($P < 0.05$; Table 6) villus height of the duodenum, jejunum and ileum than birds fed on the NC diet. The villus height of duodenum, jejunum and ileum in birds fed on the 60 mg/kg AMP-A3 diet was not different from birds fed on the NC and 90 mg/kg AMP-A3 diets. Moreover, birds given increasing concentration of the AMP-A3 had a linear ($P < 0.05$)

Table 3. *Effect of dietary supplementation of antimicrobial peptide-A3 (AMP-A3) on nutrient retention (%) in broilers*[1,2]

		AMP-A3, mg/kg				*P*-values[3]		
Item	PC	0 (NC)	60	90	SEM	T	L	Q
D 19–21 (starter)								
Dry matter	78.81[a]	74.49[c]	75.38[bc]	77.10[ab]	0.52	0.003	0.012	0.580
Crude protein	71.20[a]	65.95[c]	66.35[c]	68.18[b]	0.72	0.018	0.125	0.545
Gross energy	78.49[a]	75.41[c]	76.73[bc]	77.65[ab]	0.48	0.031	0.040	0.809
D 33–35 (finisher)								
Dry matter	74.14	71.87	72.51	72.81	0.34	0.103	0.298	0.522
Crude protein	70.31[a]	64.81[c]	65.58[bc]	68.10[ab]	0.70	0.005	0.037	0.471
Gross energy	75.11	73.55	74.15	74.37	0.34	0.184	0.279	0.759

[a,b,c]Mean values within the same row sharing a common superscript letter are not statistically different at $P < 0.05$.

[1]The dietary treatments were the following: NC: negative control (basal diet without any antimicrobials); PC: positive control (basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 and 90 mg/kg AMP-A3). The NC (diet without antimicrobials) was considered as 0 mg/kg AMP-A3.

[2]Data are means of 4 pens of 2 birds each.

[3]T: overall effect of treatments; L: linear effect of increasing AMP-A3; Q: quadratic effect of increasing AMP-A3 (0, 60 and 90 mg/kg of diet).

Table 4. *Effect of dietary supplementation of antimicrobial peptide-A3 (AMP-A3) on excreta microbial populations (Log_{10} CFU/g) in broilers*[1,2]

		AMP-A3, mg/kg				*P*-values[3]		
Item	PC	0 (NC)	60	90	SEM	T	L	Q
D 21								
TAB	8.17	8.36	8.31	8.24	0.04	0.450	0.400	0.914
Clostridium spp.	7.13[b]	7.34[a]	7.24[ab]	7.18[ab]	0.03	0.008	0.018	0.576
Coliforms	6.33[c]	6.61[a]	6.54[ab]	6.45[b]	0.03	0.035	0.013	0.732
D 35								
TAB	8.07[c]	8.42[a]	8.27[ab]	8.21[bc]	0.04	0.001	0.010	0.468
Clostridium spp.	7.09[b]	7.31[a]	7.19[ab]	7.15[b]	0.03	0.009	0.005	0.522
Coliforms	6.12[c]	6.51a	6.36[ab]	6.24[bc]	0.04	0.001	0.006	0.696

[a,b,c]Mean values within the same row sharing a common superscript letter are not statistically different at $P < 0.05$.

[1]The dietary treatments were the following: NC: negative control (basal diet without any antimicrobials); PC: positive control (basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 and 90 mg/kg AMP-A3). The NC (diet without antimicrobials) was considered as 0 mg/kg AMP-A3.

[2]Data are means of 4 pens of 2 birds each.

[3]T: overall effect of treatments; L: linear effect of increasing AMP-A3; Q: quadratic effect of increasing AMP-A3 (0, 60 and 90 mg/kg of diet).

[4]TAB: total anaerobic bacteria.

Table 5. *Effects of dietary supplementation of antimicrobial peptide-A3 (AMP-A3) on intestinal microbial populations (Log_{10} CFU/g) in broilers (d 35)*[1,2]

		AMP-A3, mg/kg				*P*-values[3]		
Item	PC	0 (NC)	60	90	SEM	T	L	Q
Ileum								
TAB	8.03	8.21	8.16	8.15	0.04	0.402	0.471	0.839
Clostridium spp.	7.04	7.23	7.10	7.08	0.03	0.058	0.097	0.373
Coliforms	5.92[c]	6.41[a]	6.28[ab]	6.13[b]	0.05	0.001	0.030	0.3654
Caecum								
TAB	8.06[b]	8.37[a]	8.23[ab]	8.17[ab]	0.04	0.035	0.058	0.686
Clostridium spp.	7.06	7.27	7.14	7.12	0.03	0.191	0.080	0.429
Coliforms	6.09[c]	6.45[a]	6.33[ab]	6.19[bc]	0.04	0.001	0.021	0.831

[a,b,c]Mean values within the same row sharing a common superscript letter are not statistically different at $P < 0.05$.

[1]The dietary treatments were the following: NC: negative control (basal diet without any antimicrobials); PC: positive control (basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 and 90 mg/kg AMP-A3). The NC (diet without antimicrobials) was considered as 0 mg/kg AMP-A3.

[2]Data are means of 4 pens of 2 birds each.

[3]T: overall effect of treatments; L: linear effect of increasing AMP-A3; Q: quadratic effect of increasing AMP-A3 (0, 60 and 90 mg/kg of diet).

[4]TAB: total anaerobic bacteria.

Table 6. *Effect of dietary supplementation of antimicrobial peptide-A3 (AMP-A3) on small intestinal morphology in broilers (d 35)*[1,2]

		AMP-A3, mg/kg				*P*-values[3]		
Item	PC	0 (NC)	60	90	SEM	T	L	Q
Villus height, μm								
Duodenum	1711[a]	1630[b]	1656[ab]	1696[a]	13.35	0.006	0.011	0.709
Jejunum	1195[a]	1117[c]	1137[bc]	1183[b]	11.58	0.021	0.033	0.590
Ileum	616[a]	509[c]	550[bc]	594[ab]	14.24	0.001	0.003	0.901
Crypt depth, μm								
Duodenum	443	448	487	473	10.30	0.423	0.442	0.353
Jejunum	354	364	375	350	8.63	0.780	0.600	473
Ileum	230	233	225	227	7.55	0.830	0.821	0.821

[a,b,c]Mean values within the same row sharing a common superscript letter are not statistically different at $P < 0.05$.

[1]The dietary treatments were the following: NC: negative control (basal diet without any antimicrobials); PC: positive control (basal diet + 15 mg avilamycin/kg diet) and AMP-A3 (basal diet supplemented with 60 and 90 mg/kg AMP-A3). The NC (diet without antimicrobials) was considered as 0 mg/kg AMP-A3.

[2]Data are means of 4 pens of 2 birds each.

[3]T: overall effect of treatments; L: linear effect of increasing AMP-A3; Q: quadratic effect of increasing AMP-A3 (0, 60 and 90 mg/kg of diet).

increase in villus height of the duodenum, jejunum and ileum. Dietary treatments had no effects on crypt depth of the duodenum, jejunum and ileum.

DISCUSSION

The antimicrobial activity of natural AMPs can be improved by designing analogue peptides by

modifying the structural properties of the natural peptides (Maloy and Kari, 1995; Javadpour *et al.*, 1996). Several attempts have been made to improve the antimicrobial activity against bacterial cells while eliminating the cytotoxicity against mammalian cells by flexible region or chain length deletion, amino acid substitution and changing the net charge, hydrophobicity and α-helicity of natural AMPs (Shin *et al.*, 1997; Oh *et al.*, 2000; Dathe *et al.*, 2001). In the present study, we used a novel peptide (AMP-A3), an analogue of the antimicrobial peptide HP (2–20) designed by substitution of amino acid tryptophan for the hydrophobic amino acids, glutamine and aspartic acid (Lee *et al.*, 2002), and its effects on growth performance, nutrient retention, excreta and intestinal microflora and small intestinal morphology in broilers were evaluated when used as a substitute to antibiotics.

Beneficial effects of dietary supplementation of avilamycin and increasing concentrations of the AMP-A3 on growth performance of broilers observed in present experiment is in good agreement with data reported by Ohh *et al.* (2009), who observed linear improvement in overall weight gain of birds given diets supplemented with increasing levels of AMP obtained from *Solanum tuberosum*. In some of the previous studies, it was reported that broilers given diets supplemented with swine gut AMPs had greater weight gain and FCR than birds given non-supplemented diets (Bao *et al.*, 2009; Wang *et al.*, 2009). Similarly, Ma *et al.* (2004) reported that AMPs can stimulate growth performance in laying hen. The lack of an effect on average daily feed intake of supplementing diets with AMP-A3 in the present experiment is consistent with the data reported in previous experiments (Jin *et al.*, 2008; Bao *et al.*, 2009; Ohh *et al.*, 2009, 2010). In contrast to present results, no effects of dietary supplementation with AMPs on growth performance were observed in the studies by Greiner *et al.* (2004) and Shan *et al.* (2007). This inconsistency in results might be due to variation in type of AMPs used, level of dietary supplementation or mode of action of the AMPs. In this experiment, as in others (Mallet *et al.*, 2005; Denev, 2006; Ohh *et al.*, 2009), broilers given a diet containing antibiotics had greater overall BW gain. The increased gain observed in birds fed on diets supplemented with avilamycin or increasing concentrations of AMP-A3 in the present study might be due to greater nutrient retention, lower number of harmful microbes in the intestine and excreta and improved small intestinal morphology. Our results indicate that dietary supplementation of AMP-A3 can affect the growth performance of broilers and should be taken into account in diet formulation as an antimicrobial growth promoter.

The improved retention of DM, CP and GE in broilers fed on a diet supplemented with avilamycin or increasing concentrations of AMP-A3 observed in present study is consistent with the data reported by Ohh *et al.* (2009, 2010). Contrary to the present findings, Jin *et al.* (2009) reported no effects of dietary supplementation of the potato AMP on apparent digestibility of DM and CP in weanling pigs. Improved nutrient retention in broilers fed on diets supplemented with AMPs might be due to modulation of gut environment, improvement of beneficial intestinal microbial balance, improved small intestinal morphology or stimulation of the mucosal immune system (Jin *et al.*, 2008; Tang *et al.*, 2009; Ohh *et al.*, 2010). On the other hand, greater nutrient retention with avilamycin supplementation might be due to increased nutrient availability for absorption and growth via suppression of growth and metabolic activities of harmful gut microbiota with simultaneous alteration in intestinal morphology, intestinal epithelium thickness and epithelial cell turnover (Miles *et al.*, 2006; Mountzouris *et al.*, 2010; Shim *et al.*, 2010). In our study, the retention of nutrients in birds fed on diets supplemented with avilamycin or 90 mg/kg AMP-A3 were comparable, which indicates the potential of 90 mg/kg AMP-A3 as an antimicrobial in broiler diets.

In the present experiment, dietary supplementation of 90 mg/kg AMP-A3 or avilamycin to broiler diets reduced harmful microflora like excreta and intestinal TAB and excreta *Clostridium* spp. In line with the present experiment, Ohh *et al.* (2010) also reported that broilers given avilamycin or potato AMPs had reduced excreta and caecal coliforms and *Clostridium* spp. count. The AMPs affect the host animal in a positive way by improving its intestinal balance and creating gut micro-ecological conditions that suppress harmful microorganisms like *Clostridium* and coliforms and by favouring beneficial microorganisms like *Lactobacillus* and *Bifidobacterium* (Wang *et al.*, 2007; Jin *et al.*, 2008; Tang *et al.*, 2009; Ohh *et al.*, 2010). Our results suggest that the AMP-A3 has potential for suppressing harmful intestinal microflora in broilers and can be used as a potential alternative to antibiotics.

Small intestinal morphology including villus height and crypt depth of duodenum, jejunum and ileum is indicative of gut health. Therefore, it was important to analyse the morphology of broiler's intestine to establish possible mechanism of growth promotion. In this experiment, supplementation of broiler diets with avilamycin or increasing concentrations of the AMP-A3 increased villus height of the duodenum, jejunum and ileum, suggesting an increased epithelial cell turnover. In line with the present study, Bao *et al.* (2009) reported increased villus height of

duodenum and jejunum in broiler chickens given diets supplemented with AMP isolated from pig small intestine. Liu *et al.* (2008) reported that birds given a diet supplemented with AMP had increased villus height in the duodenum and jejunum but not in the ileum. Increased villus height of the jejunum and ileum was also reported in weanling pigs given a diet supplemented with the AMPs lactoferricin and lactoferrampin (Tang *et al.* 2009). Increased villus height suggests an increased surface area capable of greater absorption of available nutrients (Caspary, 1992). The villus crypt is considered a villus factory and deeper crypts enhance tissue turnover to permit renewal of the villus required in response to normal sloughing or inflammation from pathogens and high demand for tissue in growing animals (Yason *et al.* 1987). Increased villus height is 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). The histomorphological changes in the small intestine of the broiler chickens reported in the present experiment demonstrated the regulative activity of AMP-A3 in improving the structure of the intestine and promoting nutrient retention and growth performance.

In conclusion, the results obtained in the present study indicated that dietary supplementation with 90 mg/kg AMP-A3 has the potential to improve growth performance, nutrient retention, intestinal morphology and reduce pathogenic bacteria in excreta and intestine of broilers and can be used as a potential antimicrobial growth promoter. However, further studies are required to identify the exact mechanism of action of AMP-A3 that underlies these observations.

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