DR-1934
[1-5]

RESEARCH ARTICLE Asian Journal of Dairy and Food Research, Volume Issue : ()

Effects of Antimicrobial Peptide Supplementation on Immune Response and Immune Organ Index in Broiler Chicks

T. Vaani Shreeya[1], R. Karunakaran[1], P. Raja[2], C. Bandeswaran[1]

10.18805/ajdfr.DR-1934

ABSTRACT

Background: Misuse of antibiotics has led to bacterial antibiotic resistance and antibiotic residues in meat and egg. The aim of this study was to assess the effect of dietary supplementation of chicken intestinal-derived antimicrobial peptide as an alternative to antibiotic growth promoters on the immune response of birds.

Methods: Immune organs of randomly selected birds were collected for immune organ index. Blood samples were collected for serum to measure the antibody titre against infectious bursal disease and Newcastle disease virus using ELISA. Whole blood was collected to study the cytokine gene expression (IL-17A, IFN-α, IFN-γ, TGF-β, IL-10) using Real-time PCR.

Result: Antimicrobial peptide supplementation had no significant effect (P>0.05) on the immune organ index and serum antibody Titre against infectious bursal disease and Newcastle disease virus on the 21st and 35th days of the study. However, it significantly (P<0.01) downregulated the gene expression of pro-inflammatory cytokines *viz*., IL-17A, IFN-α, IFN-γ and upregulated IL-10 expression in peripheral blood mononuclear cells. The results suggest that supplementation of antimicrobial peptide at a level of 100 mg/kg diet improved the cell-mediated immune response in broiler chicks but would not have been sufficient to enhance the humoral immune response and immune organ index in chicks.

Key words: Antimicrobial peptides, Cytokines, Humoral immunity, Infectious bursal disease, Newcastle disease, Real time PCR.

INTRODUCTION

Antimicrobial peptides (AMPs) are small biological molecules weighing less than 10 kDa and containing less than 100 amino acids. They are found in all species, ranging from insects and plants to animals and have a broad spectrum of activity against bacteria, some viruses and fungi (Meyerholz and Ackermann, 2005). AMPs are produced by leucocytes, neutrophils and epithelial lining in the environmental interface of the gastrointestinal tract, urogenital tract, tracheobronchial tree and skin. In chicken, antimicrobial peptides have been isolated from bone marrow, tongue, trachea, bursa of Fabricius, brain, kidney, testicle, ovary, male and female reproductive tract, liver, the urogenital tract, gastrointestinal tract and lung (Lynn *et al.,* 2004 and Ohashi *et al*., 2005).

Cathelicidins and defensins are important families of avian AMPs. Defensins are hydrophobic, cystine-rich, cationic antimicrobial peptides having the property of direct killing of microbes which includes bacteria, fungi, some of the enveloped viruses and most of the non-enveloped viruses and involved in immune signaling mechanisms (Selsted and Ouellette, 2005). Immunomodulatory properties of defensins have promoted the adaptive immunity by chemotaxis of T lymphocytes, monocytes, immature dendritic cells and mast cells (Niyonsaba *et al.,* 2002) to the sites of inflammation. About 14 types of β-defensins have been discovered from different tissues in chicken so far. The expression of avian β-defensin 2 is moderate, 9 is weak and 13 is strong in the small intestine of chicken.

[1]Department of Animal Nutrition, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.

[2]Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.

Corresponding Author: R. Karunakaran, Department of Animal Nutrition, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India. Email: drrkarunakaranphd@yahoo.com

How to cite this article: Vaani Shreeya, T., Karunakaran, R., Raja, P. and Bandeswaran, C. (2022). Effects of Antimicrobial Peptide Supplementation on Immune Response and Immune Organ Index in Broiler Chicks. Asian Journal of Dairy and Food Research. DOI: 10.18805/ajdfr.DR-1934.

Submitted: 06-04-2022 **Accepted:** 12-08-2022 **Online:** 25-08-2022

The misuse of antibiotics for growth promotion in poultry has led to bacterial antibiotic resistance and antibiotic residues in meat and egg (Marazuela and Bogialli, 2009). Hence, there is an urgent need to search for novel antibiotic substitutes for poultry having similar antimicrobial, immunomodulatory and growth promoting effects without inducing bacterial resistance and side effects. Probiotics, prebiotics, organic acids, phytobiotics, enzymes and antimicrobial peptides are a few of the potential alternatives to antibiotics that can be incorporated in feed. The objective of this study was to evaluate the effect of chicken intestinal AMP on humoral and cell-mediated immune response and immune organ index in broiler chicks.

MATERIALS AND METHODS

Preparation of AMP

The AMPs were isolated from chicken small intestines as described by Ma *et al*., 2004. The duodenum portion was separated from the rest of the intestine. Mucosal scrapings of the duodenum were washed with cold sterile normal saline (0.85 % NaCl) and extracted in ice-cold aqueous 5% acetic acid (1:10 w / v) overnight. Later, the extracts were placed in boiling water for 10 minutes and cooled quickly. The supernatant was collected after centrifugation at 9000 rpm for 30 minutes at 4°C and pH was adjusted to 7.0 with 1 M NaOH. Purification of supernatant was done by size exclusion column chromatography (Bio Rad, USA). Each elution was analyzed by agarose diffusion assay (Lehrer *et al*., 1991) with *E. coli* (MTCC accession number: 452) and *Salmonella* (MTCC accession number: 98) as test organisms. The active fractions with potent antibacterial activity were collected and detected by SDS-PAGE and then outsourced for confirmation of molecular mass by LC-MS (Ashraf and Azad, 2017) at Sophisticated Analytical Instruments Facility, Indian Institute of Technology, Madras. The analysis of antimicrobial peptide by LC-MS revealed a molecular mass of 3.76 kDa which closely corresponded to the molecular mass of avian β-defensin 2 (3.9 kDa) with the NCBI Accession Number: AAB30585. The peptide-rich fractions were lyophilized and stored at 4°C until the feeding trial.

Animals and experimental design

The feeding trial was carried out for 5 weeks in the *rabi* season of 2021 at the environmentally controlled poultry shed of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai. A total of 90 one-day-old broiler chicks (Cobb 430) were weighed, wing banded and randomly allocated in a completely randomized design for 3 treatments, each of which had 3 replicates of 10 broiler chicks. The dietary treatments were a maize-soybean meal-based diet as a control (T_1), control diet with 335 mg chlortetracycline (CTC) / kg (T_2) and control diet with 100 mg AMP/kg (T_3). Broiler chicks had *ad libitum* access to feed and water, with 23 hours of light per day. The average temperature during the biological trial period was 25.8°C. The standards of care used in the study were approved by Institutional Animal Ethics Committee (Approval Lr. No. 370/DFBS/IAEC/2021). Chicks were vaccinated as per the vaccination schedule given in Table 1.

Immunological assay

Sampling

Six birds from each treatment were randomly selected on the 21st day and 35th day, weighed and sacrificed. Immune organs were collected and weighed for immune organ index. Blood samples (2 ml) were collected for serum in plain vacutainers. Whole blood (4 ml) was collected in EDTA vacutainers for peripheral blood mononuclear cells (PBMCs) isolation on the 21st day.

Serum antibody titre against infectious bursal disease and newcastle disease virus

Antibody titres were analyzed by ELISA using IDEXX IBD and NDV Ab Test kits respectively as per the manufacturer's instructions. The viral antigen was pre-coated on 96 well plates. The serum (separated after centrifugation of blood samples at 3000 rpm for 5 minutes) was diluted to 500-fold with sample diluent provided in the kit and then 100 µl of diluted serum was added to the antigen pre-coated wells. 100 µl of both positive control (diluted chicken anti-NDV serum preserved with ProClin™ 150) and negative control (diluted chicken serum non-reactive to NDV preserved with ProClin™ 150) were also added to the wells and incubated for 30 minutes at room temperature (18-26°C). After incubation, the wells were washed thrice with approximately 350 µl of sterile distilled water. About, 100 µl of conjugate (HRP Goat anti-chicken antibody preserved with ProClin™ 150) was added and the plate was incubated for 30 minutes at room temperature (18-26°C). After incubation, the wells were washed thrice with approximately 350 µl of sterile distilled water and 100 µl of TMB substrate was added to wells in darkness and left at room temperature for 15 minutes. The reaction was stopped by adding 100 µl of stop solution to each well. The OD values were recorded at 650 nm using an ELISA plate reader. Each sample was assayed in duplicate. Results were expressed in terms of $\log_{10}$ titre values.

$$S/P = (\text{Sample mean-NC})/(\text{PC-NC})$$

$$\text{Log}_{10}\ \text{Titre} = 1.09*(\log_{10} S/P) + 3.36$$

Where,
NC is negative control and PC is positive control.

Immune organ index

Spleen, bursa and thymus were collected and weighed to calculate the immune organ index as per the formula given below:

$$\text{Immune organ index (g/kg)} = \frac{\text{Immune organ weight (g)}}{\text{Live weight before slaughtering (kg)}}$$

Gene expression of cytokines in Peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were isolated as described by Kaiser *et al.* (2006) from the whole blood by density gradient centrifugation. The High-Capacity cDNA Reverse Transcription Kit (Takara Kit) was used for the synthesis of cDNA from RNA as per the manufacturer's instructions. The cDNA synthesis cycle conditions were 37°C for 15 minutes followed by 85°C for 5 seconds. Real-time PCR analysis was performed for the expression of genes (IL-17A, IFN-α, IFN-γ, TGF-β, IL-10) with 16s rRNA gene as the housekeeping gene. Primers used for Real-Time PCR are presented in Table 2. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method and normalized using the housekeeping gene expression level. Gene expression was measured by 7500 Fast Real-Time PCR (Applied Biosystem Inc, CA, USA) with

SYBR PREMIX ex TAQ™ (Perfect Real Time, Takara, Shiga, Japan). The thermal cyclic conditions used in Real-Time PCR were as follows: 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 15 seconds and 72°C for 35 seconds.

Statistical analysis

Experimental data were analyzed by one-way ANOVA using SPSS v.20.0 statistical package and means of different treatments were compared using Duncan's multiple range test. The obtained results were expressed as mean± standard deviation.

RESULTS AND DISCUSSION

The effect of supplementation of AMP on serum antibody titres and immune organ index are presented in Tables 3 and 4, respectively. Supplementation of AMP did not significantly alter the serum antibody titres against infectious bursal disease and Newcastle disease virus on the 21st and 35th days of the study. These findings were in agreement with the findings of Yoon *et al*. (2014) who reported no significant difference in serum antibody titres in weanling piglets fed with 60 mg/kg AMP. Maintenance of normal humoral response in the AMP supplemented group is attributed to the antiviral immunity provided by AMP and also the suppression of Th1 cells and stimulation of Th2 cells required for humoral immunity. No significant difference (P>0.05) was observed in the immune organ index among all the treatments. This might be due to the broad spectrum of activity of AMP which not only maintains the humoral immune response, but also reduces the proinflammatory cytokines and antigen-presenting cells activity resulting in restriction of the triggering mechanisms for increased immune cells production, thereby regulating the immune homeostasis. However, in contrast to the present findings, Bai *et al*. (2019) reported improved immune organ index and serum Newcastle disease antibody titre which would have been due to the supplementation of a higher dose of AMP (300-600 mg/kg).

The present study revealed for the first time the expression pattern of proinflammatory (IL-17A, IFN-α and IFN-γ) and anti-inflammatory (TGF-β and IL-10) cytokines in PBMCs of broiler chicken on dietary supplementation of AMP. PBMCs are considered potential immune cellular systems that harbor cytokines (Davila and De Los Rios, 2019). They are sensitized by circulating antigens that gain entry through the intestinal tract and modulate the cytokine expression depending on their concentration. Several studies disclose the importance of defensins in innate and humoral immune responses against a broad spectrum of microbes (Oppenheim *et al*., 2003; Yurong *et al*., 2006). AMPs enhance the immune response by modulating the cytokine expression and activation of T and B cell production. In the present study, mRNA expression of proinflammatory cytokines *viz*., IL-17A, IFN-α and IFN-γ (Fig 1A, 1B and 1C, respectively) decreased and anti-inflammatory cytokine, IL-10 (Fig1E) increased. There was no significant change in the expression pattern of TGF-β (Fig 1D) as compared to the control group. IL-10, secreted by B lymphocytes regulates the B cell maturation and antibody production. Also, IL-10 suppresses the activity of natural killer cells and macrophages as well as downregulates the expression of

Table 1: Vaccination schedule.

Age	Name of the Vaccine	Route of administration
0 day	Marek's disease vaccine	Subcutaneous
7th day	Ranikhet or Newcastle disease vaccine – *RDV- F strain*	Intraocular
14th day	Infectious Bursal Disease vaccine (*Georgia strain*)	Intraocular
21st day	Ranikhet or Newcastle disease vaccine - *Lasota strain*	Drinking water

Table 2: The sense and antisense primer sequences of cytokines and housekeeping gene used for Real-time PCR.

Gene	Orientation	Primers sequences (5′ - 3′)	Product size (bp)
IL-17A	Forward	AGATGCTGGATGCCTAACCC	188
	Reverse	ACTGGGCATCAGCAACCAAG	
IFN-α	Forward	CCAGCACCTCGAGCAAT	133
	Reverse	GGCGCTGTAATCGTTGTCT	
IFN-=γ	Forward	ATCATACTGAGCCAGATTGTTTCG	140
	Reverse	TCTTTCACCTTCTTCACGCCAT	
TGF-β	Forward	CGGGACGGATGAGAAGAA	141
	Reverse	TCGGCGCTCCAGATGTAC	
IL-10	Forward	CACGCGGAGGGCGTTAAA	186
	Reverse	CAGGTGAAAGTCAGCCCGT	
16S	Forward	GTAACGCAAGCGATCNCG	130
	Reverse	AACCGCGACGCTTTCCAA	

Reference: Xie *et al*. (2020).

pro-inflammatory cytokines, MHC II molecules and antigen-presenting cells activity thereby, playing an immunomodulatory role (Trinchieri, 2007). In this study, AMP supplementation improved the expression of IL-10 that led to the suppression of proinflammatory cytokines and Th1, while stimulated Th2 cell activity. TGF-β regulates cell proliferation, differentiation and growth. It has the ability to inhibit macrophage activation, T cell and B cell apoptosis as well as reduces the expression of inflammatory mediators such as TLR-4, IL-1, IL-6 and IFN-γ to maintain the immune homeostasis (Bickerstaff and Orosz, 2002). The current study results revealed no significant changes in the mRNA expression pattern of TGF-β gene and the basal level of gene expression required for regulation of cell cycle activity was maintained similar to the control group. IL-17A enhances the production of cytokines (IL-6, G-CSF, GM-CSF) chemokines (IL8, $CXCL_1$ and $CXCL_5$), neutrophil chemotaxis, antibody production and activation of T cells (Iwakura *et al*., 2008). In the present study, supplementation of AMP downregulated the mRNA expression of IL-17A mediated by IL-10. IFN-α (Type I interferon) exhibits a

Table 3: Effect of supplementation of antimicrobial peptide on serum antibody titers against infectious bursal disease and newcastle disease virus at various stages (Mean±SD).

Stage	Control group (T_1)	CTC (T_2)	AMP (T_3)
	Infectious bursal disease		
21st day	1.94±0.5	2.38±0.7	2.09±0.43
35th day	3.16±0.19	2.85±0.42	2.99±0.53
	Newcastle disease		
21st day	2.88±0.35	3.06±0.27	3.14±0.23
35th day	2.74±0.32	2.65±0.3	2.8±0.2

Values in a row did not differ significantly (P>0.05).
CTC: Chlortetracycline; AMP: Antimicrobial peptide.

Table 4: Effect of supplementation of antimicrobial peptide on the immune organ index of broiler chickens (Mean±SD).

Stage	Indicator	Control group (T_1)	CTC (T_2)	AMP (T_3)
21st day	Spleen (g/kg)	0.96±0.19	0.97±0.2	1.04±0.1
	Thymus (g/kg)	3.49±0.38	3.58±0.42	3.8±0.77
	Bursa (g/kg)	1.65±0.43	1.91±0.41	2.00±0.76
35th day	Spleen (g/kg)	1.02±0.21	0.97±0.22	1.34±0.39
	Thymus (g/kg)	2.85±1.14	2.97±0.86	3.30±0.76
	Bursa (g/kg)	1.69±0.47	1.68±0.66	1.74±0.66

Values in a row did not differ significantly (P>0.05).
CTC: Chlortetracycline; AMP: Antimicrobial peptide.

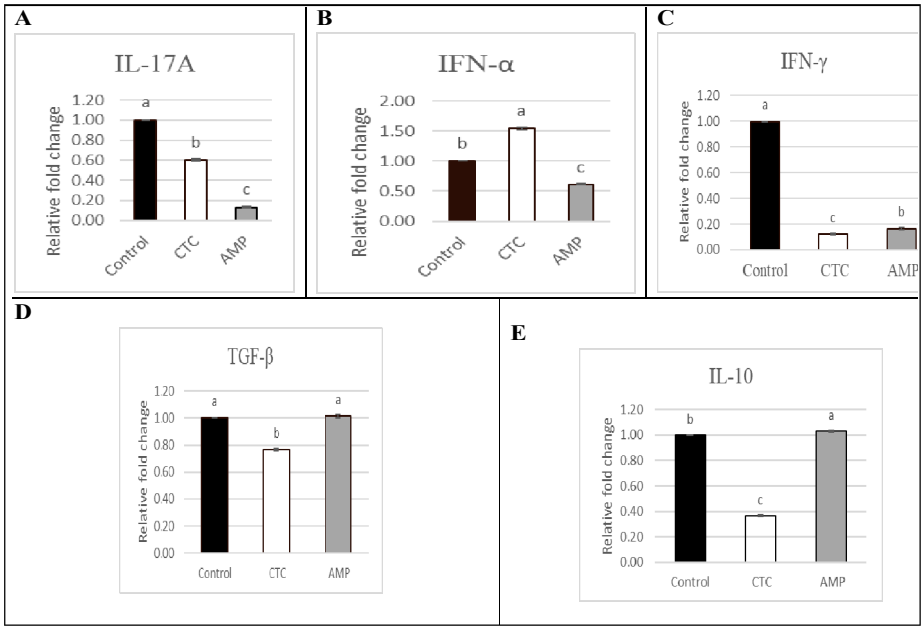

Fig 1: Effect of supplementation of antimicrobial peptide on different cytokines of broiler chickens. (A) The effect of different treatments on IL-17A. (B) The effect of different treatments on IFN-α. (C) The effect of different treatments on IFN-γ. (D) The effect of different treatments on TGF-β. (E) The effect of different treatments on IL-10. In all the figures, the horizontal axis represents different groups. Values with different superscripts differ significantly (P<0.01).
CTC: chlortetracycline; AMP: antimicrobial peptide.

pleiotropic effect on the cells including antiviral immunity, innate immune response and modulating the cell fatality (survival/apoptosis). IFN-γ (Type II interferon) is a potential macrophage-activating factor and is also involved in the activation and regulation of innate and adaptive immune responses (Plachy *et al*., 1999). AMP supplementation downregulated the mRNA expression of IFN-α and IFN-γ in this study due to the antiviral defenses provided and enhanced cell protection by a reduction in the expression of interferon-stimulated genes, immunoproteasome activity and activation of other immune cells that prevent apoptosis.

CONCLUSION

Antimicrobial peptide supplementation had no significant ($P>0.05$) effect on immune organ index and serum antibody titres against infectious bursal disease and Newcastle disease virus measured on 21st and 35th days of the study. It significantly ($P<0.01$) downregulated the expression of pro-inflammatory cytokines (IL-17A, IFN-α, IFN-γ) and upregulated the expression of anti-inflammatory cytokine IL-10 on 21st day of the study. Hence, it is concluded that supplementation of AMP, at a dietary concentration of 100 mg/kg enhanced the cell-mediated immune response of broiler chicks mediated through cytokines.

ACKNOWLEDGMENT

The authors thank Tamil Nadu Veterinary and Animal Sciences University for providing the necessary facilities to carry out the research work.

Conflict of interest: None.

REFERENCES

Ashraf, S.A. and Azad, Z.A.A. (2017). Development and validation of an UPLC-ESI-MS/MS analytical method for the determination of streptomycin and dihydrostreptomycin residues in honey. Biomedical and Pharmacology Journal. 10(4): 1983-1992.

Bai, J., Wang, R., Yan, L. and Feng, J. (2019). Co-supplementation of dietary seaweed powder and antibacterial peptides improves broiler growth performance and immune function. Brazilian Journal of Poultry Science. 21.

Bickerstaff, A. and Orosz, C. (2002). Evidence for a limited contribution of immune regulation to cardiac allograft acceptance. Human immunology. 63(10): 935-947.

Davila, J.A.A. and De Los Rios, A.H. (2019). An overview of peripheral blood mononuclear cells as a model for immunological research of Toxoplasma gondii and other apicomplexan parasites. Frontiers in Cellular and Infection Microbiology. 9. https://doi.org/10.3389/fcimb.2019.00024.

Iwakura, Y., Nakae, S., Saijo, S. and Ishigame, H. (2008). The roles of IL 17A in inflammatory immune responses and host defense against pathogens. Immunological Reviews. 226 (1): 57-79.

Kaiser, M.G., Cheeseman, J.H., Kaiser, P. and Lamont, S.J. (2006). Cytokine expression in chicken peripheral blood mononuclear cells after *in vitro* exposure to Salmonella enterica serovar Enteritidis. Poultry Science. 85(11): 1907-1911.

Lehrer, R.I., Rosenman, M., Harwig, S.S., Jackson, R. and Eisenhauer, P. (1991). Ultrasensitive assays for endogenous antimicrobial polypeptides. Journal of Immunological Methods. 137(2): 167-173.

Lynn, D.J., Higgs, R., Gaines, S., Tierney, J., James, T., Lloyd, A.T. and O'Farrelly, C. (2004). Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. Immunogenetics. 56(3): 170-177.

Ma, W.M., She, R.P., Peng, F.Z., Jin, H. and Hu, Y.X. (2004). The preparation and patial characterization of an antimicrobial peptide from the small intestine of pig. Sci. Technol. Eng. 3: 202-205.

Marazuela, M.D. and Bogialli, S. (2009). A review of novel strategies of sample preparation for the determination of antibacterial residues in foodstuffs using liquid chromatography-based analytical methods. Analytica Chimica Acta. 645(1-2): 5-17.

Meyerholz, D.K. and Ackermann, M.R. (2005). Antimicrobial peptides and surfactant proteins in ruminant respiratory tract disease. Veterinary Immunology and Immunopathology. 108(1-2): 91-96.

Niyonsaba, F., Iwabuchi, K., Matsuda, H., Ogawa, H. and Nagaoka, I. (2002). Epithelial cell derived human β defensin 2 acts as a chemotaxin for mast cells through a pertussis toxin sensitive and phospholipase C dependent pathway. International Immunology. 14(4): 421-426.

Ohashi, H., Subedi, K., Nishibori, M., Isobe, N. and Yoshimura, Y. (2005). Expressions of antimicrobial peptide gallinacin-1,-2 and-3 mRNAs in the oviduct of laying hens. The Journal of Poultry Science. 42(4): 337-345.

Oppenheim, J.J., Biragyn, A., Kwak, L.W. and Yang, D. (2003). Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. Annals of the Rheumatic Diseases. 62: ii17-ii21.

Plachý, J., Weining, K.C., Kremmer, E., Puehler, F., Hala, K., Kaspers, B. and Staeheli, P. (1999). Protective effects of type I and type II interferons toward Rous sarcoma virus-induced tumors in chickens. Virology. 256(1): 85-91.

Selsted, M.E. and Ouellette, A.J. (2005). Mammalian defensins in the antimicrobial immune response. Nature immunology. 6(6): 551-557.

Trinchieri, G. (2007). Interleukin-10 production by effector T cells: Th1 cells show self-control. The Journal of Experimental Medicine. 204(2): 239-243.

Yoon, J.H., Ingale, S.L., Kim, J.S., Kim, K.H., Lee, S.H., Park, Y.K. and Chae, B.J. (2014). Effects of dietary supplementation of synthetic antimicrobial peptide-A3 and P5 on growth performance, apparent total tract digestibility of nutrients, fecal and intestinal microflora and intestinal morphology in weanling pigs. Livestock Science. 159: 53-60.

Yurong, Y., Yibao, J., Ruiping, S., Qingqiang, Y., Kaisong, P., Huihui, B. and Xuemei, Z. (2006). Effects of chicken intestinal antimicrobial peptides on humoral immunity of chickens and antibody titres after vaccination with infectious bursal disease virus vaccine in chicken. Archives of Animal Nutrition. 60(5): 427-435.