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Effects of Dietary Supplementation of Antimicrobial Peptide on Growth Performance, Serum Biochemistry and Intestinal Health in Broilers

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ABSTRACT

Background: The study was undertaken to determine the efficiency of supplementation of antimicrobial peptide on growth performance, serum biochemistry, intestinal morphology, antioxidant status and gene expression of cytokines and tight junction proteins in broiler chicks as an alternative to antibiotic growth promoters that impart residues in broiler meat.

Methods: The FCR was calculated by dividing the respective feed intake of chicks by weight gain during the respective period. Two broiler chicks were randomly selected from each replicate based on the average body weight of the corresponding replicate. After sacrifice, blood for serum and sections of small intestine were collected for the assessment of intestinal morphology, antioxidant activity measurement and gene expression of cytokines and tight junction proteins.

Result: The results revealed that supplementation of antimicrobial peptide significantly improved the overall weight gain of broiler chicks compared to the NC. There was a significant increase in the activities of intestinal *superoxide dismutase* and *glutathione peroxidase* in the antimicrobial peptide supplemented group. AMP significantly modulates the gene expression of intestinal cytokines (IL-17A, IFN-α, IFN-γ and IL-10) and tight junction protein genes. These results indicate that antimicrobial peptide supplementation had beneficial effects on growth performance and intestinal health in broilers.

Key words: Antimicrobial peptide, Avian â-defensin, Broilers, Cytokines, Tight junction proteins.

INTRODUCTION

Antibiotics have been widely used for the growth promotion and prevention of diseases in livestock and poultry. However, the misuse of antibiotics disrupts the micro-ecological balance of the intestine and also leads to bacterial antibiotic resistance and antibiotic residues in meat and eggs (Marazuela and Bogialli, 2009). The emergence and rapid spread of antibiotic-resistant microbes poses risk for human health. Hence, there is an urgent need to search for novel antibiotic substitutes. Probiotics, prebiotics, organic acids, phytobiotics, enzymes and antimicrobial peptides (AMPs) are a few potential alternatives.

AMPs are small biological molecules (<10 kDa) containing less than 100 amino acids, found in all species, ranging from plants and insects to animals and having broad-spectrum antimicrobial activity against bacteria, some viruses and fungi (Meyerholz and Ackermann, 2005). Cathelicidins and β-defensins are the two major families of AMPs in avian species. Defensins are cysteine-rich peptides that play an important role in the innate immune system and are capable of killing a broad spectrum of pathogens. About 14 types of β-defensins have been discovered in chicken from different tissues such as bone marrow, tongue, trachea, bursa of Fabricius, brain, kidney, testicle, ovary, male and female reproductive tract, liver and the urogenital tract, gastrointestinal tract and lung so far (Lynn *et al*.,, 2004). Among these, the expression of avian β-defensin 9 is weak, 2 is moderate and 13 is strong in the small intestine of birds.

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Natural compounds such as AMPs are attractive possibilities to replace antibiotics because of their natural antimicrobial, immunomodulatory properties and a low tendency for the development of bacterial resistance. Consequently, the present study was undertaken to evaluate the effect of chicken intestinal AMPs on growth performance and intestinal health in broiler chicks.

MATERIALS AND METHODS

Chicken intestinal AMP

The AMPs were extracted from chicken small intestines, as described by Ma *et al*., (2004). Size exclusion column chromatography was used to purify the AMP rich supernatant (Bio Rad, USA). The SDS-PAGE analysis of active fraction with potential antimicrobial activity revealed band size with a molecular mass of less than 5 kDa. The fraction was then outsourced to the Sophisticated Analytical Instruments Facility, Indian Institute of Technology, Madras for confirmation of molecular mass by LC-MS (Ashraf and Azad, 2017). The antimicrobial peptide had a molecular mass of 3.76 kDa, which closely corresponded to the molecular weight of avian β-defensin 2 (3.9 kDa) with the NCBI Accession Number: AAB30585. The peptide-rich fractions were lyophilized and kept at 0°C until further use.

Animals, diet and experimental design

A total of 90 one-day-old healthy broiler chicks (Cobb 400) were randomly allotted to three dietary treatments based on body weight. There were 3 replicates in each treatment with 10 birds per replicate. The dietary treatments included, negative control (NC; basal diet), positive control (PC; basal diet supplemented with 335 mg chlortetracycline/kg) and antimicrobial peptide groups (AMP; basal diet supplemented with 100 mg AMP/kg). The experimental diets were formulated based on three phases: pre-starter (day 1 to 7), starter (day 8 to 21) and finisher (day 22 to 35). Birds had *adlibitum* access to feed and water during the trial. The standards of care used in the study were approved by Institutional Animal Ethics Committee (Approval Lr. No. 370/DFBS/IAEC/2021). All the diets were formulated to meet BIS (2007) broiler nutrient recommendations. The feed ingredients and nutrient levels of the basal diet in three phases were presented in Table 1.

Sample preparation and measurements

Body weights of individual birds were measured on days 1, 7, 14, 21, 28 and 35 and the weight gain was calculated from that data. The FCR was calculated by dividing the respective feed intake of the chicks by weight gain during the respective period. On day 35th the intestinal tissues were collected from the midpoint of the duodenum, jejunum and ileum for the assessment of intestinal morphology and a part of intestinal tissue was used for measurement of antioxidant activity. And remaining intestinal tissue was immediately processed for RNA isolation.

Serum biochemical indicators

The serum samples collected on day 21st were analyzed for *Alanine transaminase* (ALT), Albumin, *Aspartate*

Table 1: Feed ingredients and nutrient levels of basal diets at different phases of growth.

Ingredients (%)	Pre-starter diet (1-7 days)	Starter diet (8-21 days)	Finisher diet (22-35 days)
Maize grain	44.29	50.60	52.41
De-oiled rice bran	3.00	0.00	1.86
Soybean meal	41.90	39.90	34.50
Palm oil	4.50	5.10	6.70
Common salt	2.20	0.30	0.30
Calcite	0.00	0.00	0.10
Di-calcium phosphate	1.20	1.20	1.20
Mineral mixture[1]	2.00	2.00	2.00
Vitamin premix[2]	0.25	0.25	0.25
DL-methionine	0.21	0.20	0.18
Choline chloride 60%	0.15	0.15	0.15
L-Threonine	0.10	0.10	0.15
Coccidiostat	0.05	0.05	0.05
Liver tonic	0.04	0.04	0.04
Toxin binder	0.10	0.10	0.10
Antioxidant	0.01	0.01	0.01
Total	100.00	100.00	100.00
Calculated nutrient levels			
CP (%)	23.01	21.98	20.12
ME (kcal/kg)	3010	3102	3200
Lysine (%)	1.56	1.48	1.31
Methionine (%)	0.60	0.58	0.55

The antimicrobial peptide and chlortetracycline were added to the diets by using a portion of the basal diet as carrier.

[1]Mineral mixture provided per kilogram of diet: Copper 12 mg, Iodine 1.6 mg, Iron 80 mg, Manganese 100 mg, Zinc 80 mg.

[2]Vitamin premix provided per kilogram of diet: Vitamin A 10000 IU, Vitamin D_3 3000 IU, Vitamin E 40 IU, Vitamin K_3 1.5 mg, Vitamin B_{12} 0.01 mg, Biotin 0.15 mg, Folic acid 1 mg, Niacin 40 mg, Pantothenic acid 15 mg, Pyridoxine 5.5 mg, Riboflavin 6.5 mg, Thiamine 2.5 mg.

aminotransferase (AST), Glucose, Total protein, Uric acid and *Alkaline Phosphatase* (ALP) using A15 Biosystem auto analyzer.

Intestinal morphology

The intestinal tissues were processed and stained with hematoxylin and eosin (Uni *et al*., 1998) and histological indices measured by an image analyzer software (Alpha imager hp version 5.0) to assess the intestinal length to crypt depth (V/C) ratio.

Intestinal antioxidant capacity

Intestinal tissue was crushed and 10% (w/v) homogenate was prepared with 0.05M PBS (pH 7.4). The homogenate was centrifuged and used for the estimation of malondialdehyde (MDA), *superoxide dismutase* (SOD), *catalase*, *glutathione peroxidase* (GSH-Px) and total antioxidant capacity (T-AOC). Malondialdehyde was determined by the thiobarbituric acid reactive substances method as described by Ohkawa *et al*. (1979). The GSH-Px activity was determined by the method of Rotruck *et al*., (1973). SOD activity was determined by the method described by Marklund and Marklund (1974). *Catalase* activity was assayed according to the method of Caliborne and Greenwald (1985). The DPPH radical scavenging assay was performed to determine the T-AOC as described by Xing *et al*. (2015).

Table 2: The sense and antisense primer sequences of cytokines, intestinal tight junction proteins and reference 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	
MUC-2	Forward	TTCATGATGCCTGCTCTTGTC	93
	Reverse	CCGTAGCCTTGGTACATTCTTGT	
ZO-1	Forward	GCCTGAATCAAACCCAGCAA	197
	Reverse	TATGCGGCGGTAAGGATGAT	
Claudin-3	Forward	GAAGGGCTGTGGATGAACTG	221
	Reverse	GAGACGATGGTGATCTTGGC	
16S	Forward	GTAACGCAAGCGATCNCG	130
	Reverse	AACCGCGACGCTTTCCAA	

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

Table 3: Effects of supplementation of antimicrobial peptide on growth performance of broiler chicken (Mean$^{\#}$±SD).

Stage	Indicator	NC	PC	AMP	P-value
Pre-starter (1-7 days)	Body weight gain (g)	112.72±19.87	112.76±20.98	113.43±28.68	0.99
	Average feed intake (g)	150.30^{a}±3.57	135.50^{c}±1.95	144.40^{b}±6.94	0.00**
	FCR (g/g)	1.38±0.26	1.25±0.28	1.39±0.52	0.28
Starter (8-21 days)	Body weight gain (g)	788.69±101.11	765.53±102.54	812.36±98.49	0.28
	Average feed intake (g)	1081.90^{b}±23.99	1086.90^{b}±52.27	1123.75^{a}±93.73	0.04*
	FCR (g/g)	1.39±0.19	1.44±0.21	1.41±0.23	0.68
Finisher (22-35 days)	Body weight gain (g)	1035.13^{b}±140.25	1136.92^{a}±102.41	1161.63^{a}±142.91	0.00**
	Average feed intake (g)	2051.96±153.9	2115.71±55.56	2088.15±119.86	0.17
	FCR (g/g)	2.03^{a}±0.38	1.88^{ab}±0.17	1.82^{b}±0.22	0.03*
Overall (1-35 days)	Body weight gain (g)	1935.57^{b}±217.51	2017.17^{ab}±192.95	2088.35^{a}±192.65	0.03*
	Average feed intake (g)	3284.16±179.32	3338.11±109.48	3356.04±219.13	0.34
	FCR (g/g)	1.72±0.23	1.67±0.17	1.62±0.14	0.16
	Survival rate (%)	100.00±0.00	100.00±0.00	100.00±0.00	-

Means bearing different superscripts differ significantly (P<0.05)* (P<0.01)**.

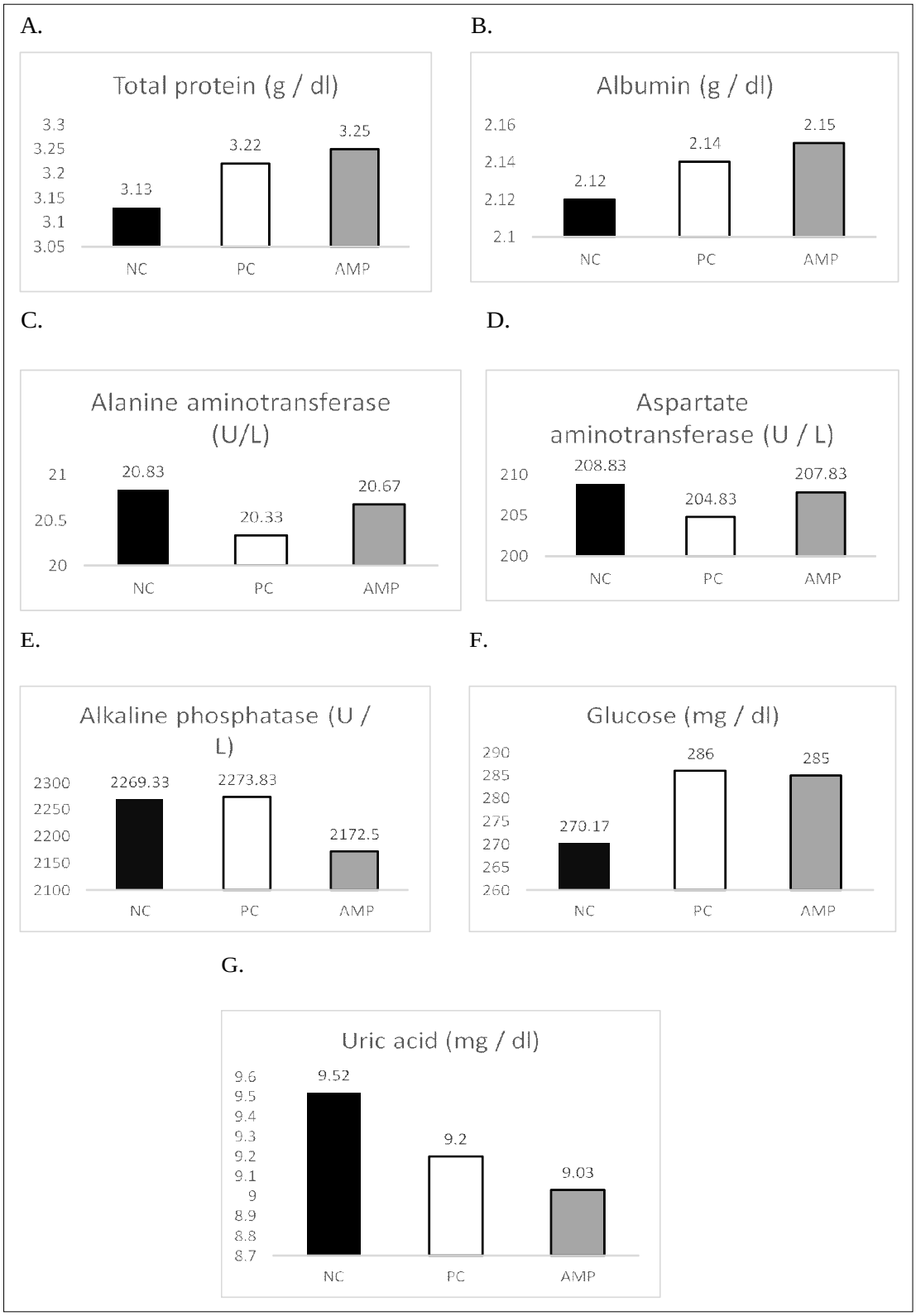

Fig 1: Effect of supplementation of antimicrobial peptide on serum biochemical indicators of 21-day old broiler chicken.

DPPH radical scavenging activity (U/ml) =

$$\frac{(\text{Absorbance of control - Absorbance of sample})}{\text{Sample volume (ml)}} \times 100$$

Gene expression analysis of cytokines and tight junction proteins

A piece (25 mg) of the intestine was ground and treated with 750 µl Trizol Reagent (Sigma) to extract the RNA as described by Kaiser *et al*. (2006). The High-Capacity cDNA Reverse Transcription Kit (Takara Kit) was used for cDNA synthesis as per the manufacturer's instructions. The expression of cytokine and tight junction protein genes (IL-17A, IFN-α, IFN-γ, TGF-β, IL-10, MUC-2, ZO-1 and Claudin-3) was measured using real-time PCR with the 16s rRNA gene as the housekeeping gene. Primers used for real-time PCR were given in Table 2. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method and normalized using the expression level of the housekeeping gene. The thermal cyclic conditions used for 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

Data were analyzed by one-way ANOVA using SPSS v.20.0 statistical package. When dietary treatment was significant (P<0.05), means were compared using the Duncan test. The results were expressed as mean±standard deviation.

RESULTS AND DISCUSSION

The effect of supplementation of AMP on growth performance is presented in Table 3. A significantly (P<0.05) higher body weight gain was observed in the AMP supplemented group during finisher and overall periods when compared to NC. FCR did not significantly (P>0.05) vary among the treatments during pre-starter, starter and overall period. These results were in agreement with Bao *et al*., (2009) who reported greater body weight gain in broilers supplemented with swine gut antimicrobial peptides. The improvement in weight gain may be because of an increase in nutrient absorption and utilization as it has been reported that AMP could improve the apparent digestibility of dry matter, crude protein, crude fat and gross energy in broilers (Wen and He, 2012). In contrast to the present findings, no effect of supplementation of AMP on growth performance (Shan *et al*., 2007), while significantly lower FCR was reported by Bai *et al*., (2019). This inconsistency in results might be due to variation in the types of AMPs supplemented and the form of feed (Landy *et al*., 2020). The present study revealed no difference in the overall weight gain and FCR between PC and AMP groups which makes it evident that AMP could safely replace antibiotic growth promoters in the broiler diet.

AMP revealed no significant (P>0.05) difference in serum biochemical indicators (glucose, total protein, albumin, ALT, AST, ALP and uric acid) on day 21 is presented in Fig 1. These findings were in agreement with Xie *et al*., (2020) who reported that supplementation of AMPs (100mg plectasin and cecropin/kg) with and without cinnamaldehyde had no effect on serum ALT, AST, albumin, total protein, glucose and uric acid.

AMP revealed no significant (P>0.05) difference in the villus length of the duodenum of 21 days old broiler chicken is presented in Fig 2. However, there was a significant (P<0.05) increase in the villus length in the AMP group

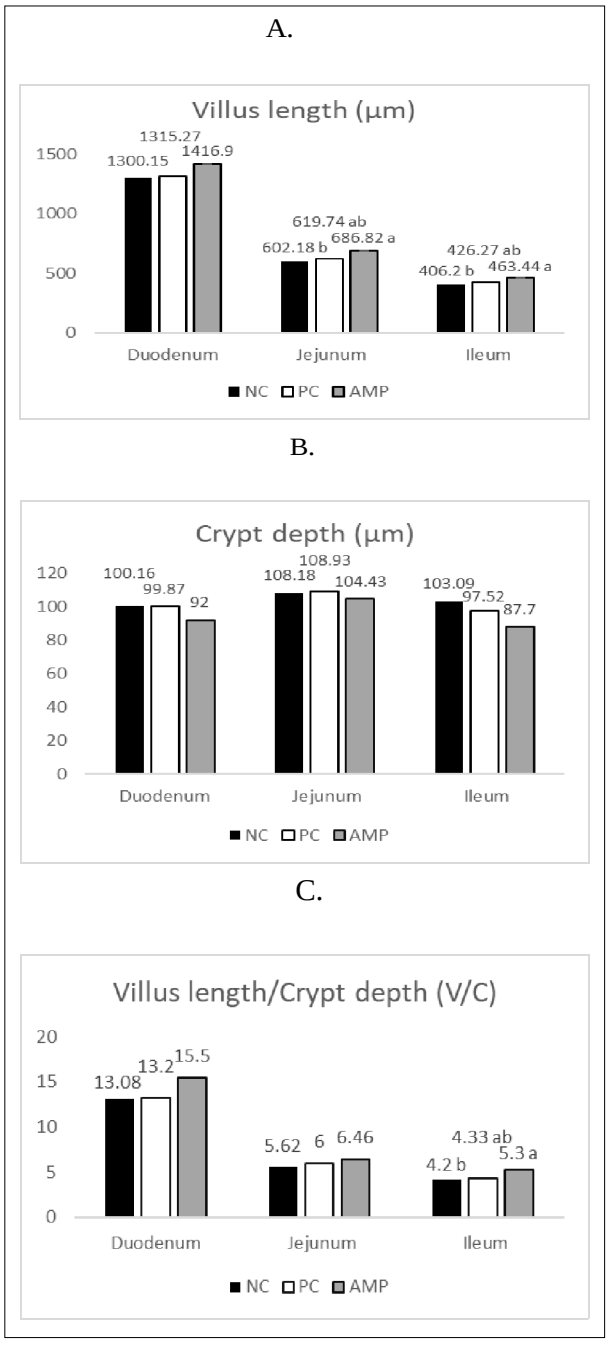

Fig 2: Effect of supplementation of antimicrobial peptide on intestinal morphology of 21-day old broiler chicken. Means bearing different superscripts differ significantly (P<0.05).

compared to NC in jejunum and ileum. A significant increase (P<0.05) in villus length to crypt depth ratio was noticed in AMP group compared to the NC in the ileum. In line with the present study, Bao *et al*., (2009) reported a significant increase in jejunal villus length of AMP supplemented birds. Increased villus length suggests an increased surface area for greater absorption of the available nutrients (Caspary, 1992).

The dietary supplementation of AMP significantly (P<0.01) improved the *glutathione peroxidase* activity when compared to PC and NC. The SOD activity was significantly (P<0.05) higher in the AMP group compared to the NC. There

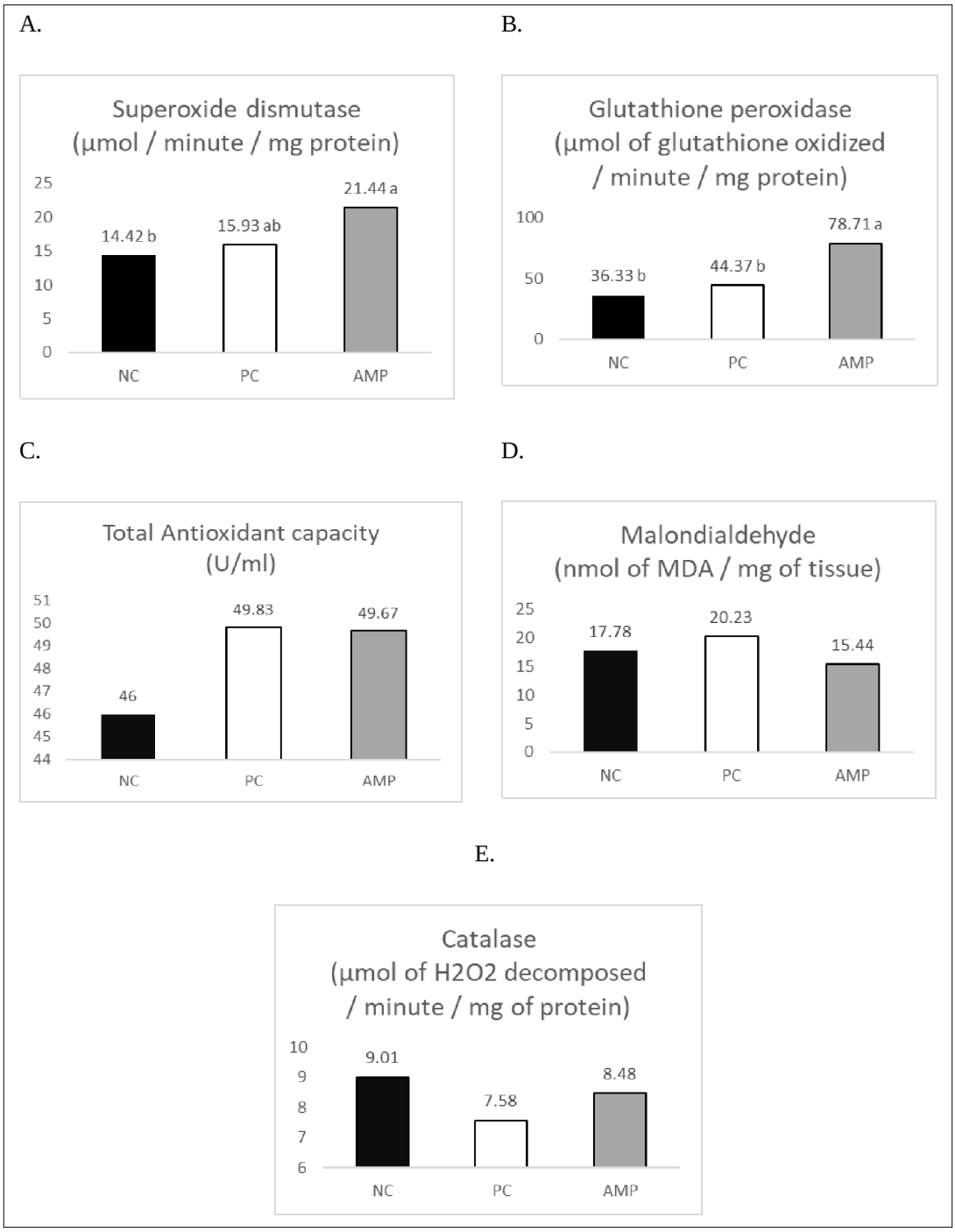

Fig 3: Effect of supplementation of antimicrobial peptide on intestinal antioxidant capacity of 21-day old broiler chicken. Means bearing different superscripts differ significantly (P<0.05).

was no significant (P>0.05) difference in the total antioxidant capacity, malondialdehyde and *catalase* activity among all the treatment groups (Fig 3). These findings were in agreement with Xie *et al*., (2020) who reported that supplementation of AMPs (100 mg plectasin and cecropin/kg) with and without cinnamaldehyde had no effect on malondialdehyde, catalase and total antioxidant capacity.

AMP significantly (P<0.01) lowered the gene expression of pro-inflammatory cytokines *viz*., IL-17 A, IFN-α, IFN-γ, anti-inflammatory cytokine IL-10 and tight junction proteins *viz*.,

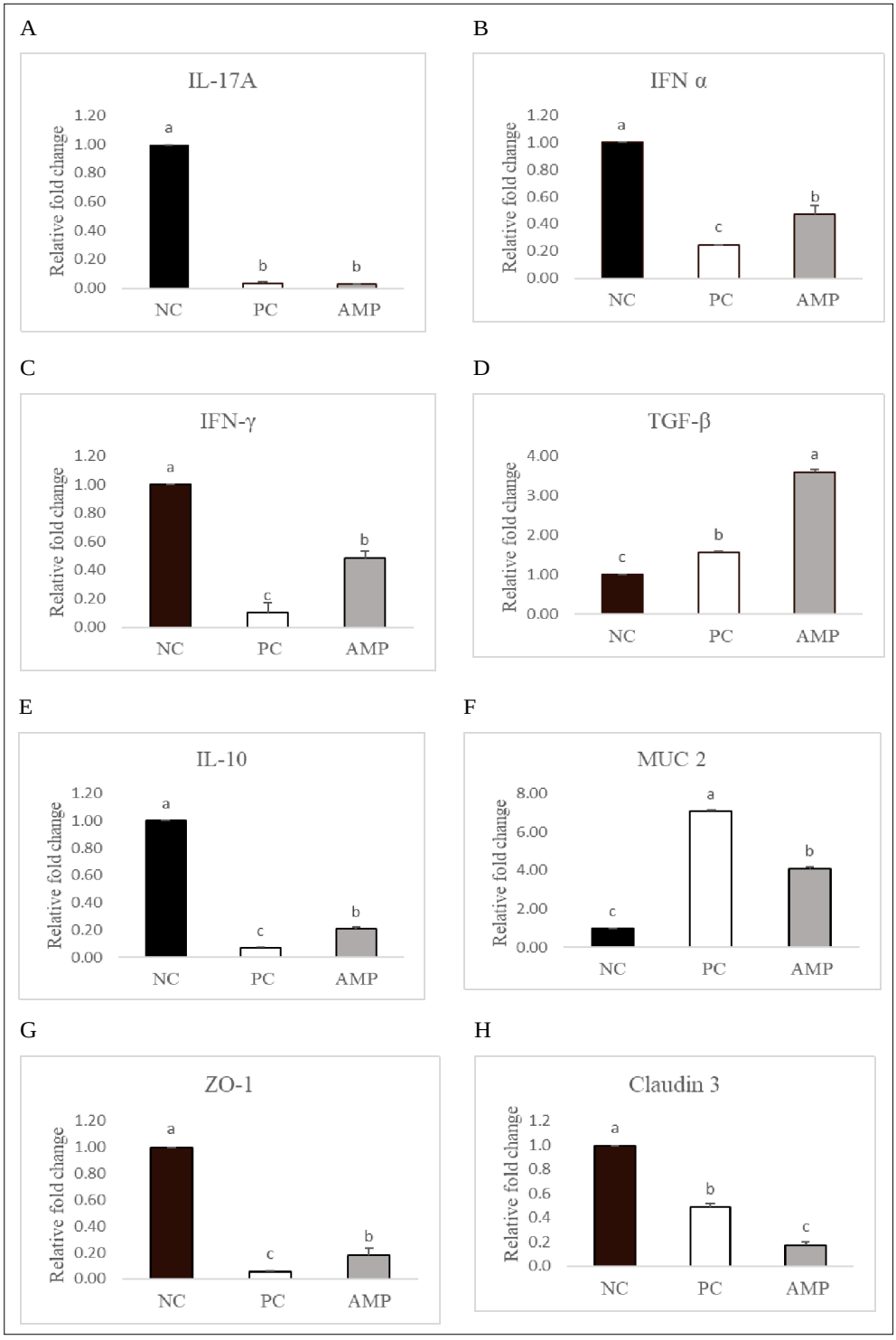

Fig 4: Effect of supplementation of antimicrobial peptide on gene expression of cytokines and tight junction proteins of 21 day-old broiler chickens. Means bearing different superscripts differ significantly (P<0.01).

ZO-1 and Claudin-3 compared to the NC group (Fig 4). The expression of interferons and the extent of inflammation in the body have a positive correlation. As a result, there is reduced intestinal inflammation and the function of digestion and absorption of nutrients is retained. The above findings were in agreement with Xie *et al*., (2020) who reported a reduction in the expression of IL-17 A, IFN-α and Zhang *et al*., (2021) who reported reduced expression of IL-17A, IFN-α and IFN-γ in the intestine on AMP supplementation. AMP significantly increased the expression of anti-inflammatory cytokine TGF-γ and intestinal tight junction protein MUC-2. TGF-γ can increase tight junction protein expression in intestinal epithelial cells, maintain transmembrane potential balance and strengthen the intestinal mucosal barrier (Howe *et al*., 2005). The upregulation of MUC-2 gene by AMP indicated that MUC-2 provided a protective barrier between the epithelial surfaces and the gut lumen (Allen *et al*., 1998).

CONCLUSION

Supplementation of AMP improved the weight gain of broiler chicks by 7.32%. There was a 5.81% rise in FCR in the AMP group, which was however not statistically significant. AMP supplementation increased the villus length of jejunum, ileum and V/C ratio of ileum and intestinal *Superoxide dismutase* and *Glutathione peroxidase* in the AMP group. AMP supplementation modulates the expression of cytokines and intestinal tight protein genes. Hence, this study concludes that antimicrobial peptide improved the growth performance and intestinal health in broilers and has the potential for application in broiler diets as an alternative to antibiotic growth promoters.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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