A Meta-Analysis Antimicrobial Peptide Effects on Intestinal Bacteria, Immune Response and Antioxidant Activity of Broilers

ABSTRACT

This study used a meta-analysis to systematically assess the effect of antimicrobial peptide (AMP) addition on the number of bacteria, immune responses, and antioxidant activity of broilers. Database was compiled from 29 post evaluation articles that found in search engines, there were 36 experiments and 111 data. Mixed model method was used to assess the effect of AMP, with AMP addition level as fixed effect and experiment as random effect. The fixed effect was tested for linear and quadratic models. The quadratic model was retained when significant at $p < 0.05$, but turned into its corresponding linear model when insignificant. In starter phase, AMP addition decreased the number of bacteria in the ileum (coliform and total aerobic bacteria (TAB); $p < 0.05$), caecum (*Clostridium* spp., *Escherichia coli*, coliform and lactic acid bacteria (LAB); $p < 0.05$), and excreta (*Clostridium* spp.; $p < 0.1$). Similarly, the number of bacteria also declined in the ileum (*Escherichia coli*, $p < 0.05$; TAB, $p < 0.1$), caecum (LAB; $p < 0.1$), and excreta (*Clostridium* spp.; $p < 0.05$) of broilers in the finisher phase. There was significant improvement of immune response and antioxidant activity in starter broiler, as indicated by Newcastle disease (ND) antibody titer, bursal index, spleen index, and thymus index ($p < 0.05$) due to AMP addition. Variables of immunoglobulin M (IgM), cluster of differentiation 4 (CD4), ND antibody titer, bursal index, spleen index and thymus index were also significantly increased ($p < 0.05$) while superoxide dismutase activity (SOD activity) tended to increase ($p < 0.1$) in finisher broiler following the AMP addition. In short, AMP addition is able to suppress the

number of pathogenic bacteria and increase the immune response and antioxidant activity of broilers.

Key words: antimicrobial peptide, gut bacteria, immune response, meta-analysis, antioxidant activity.

INTRODUCTION

The awareness of world community on the need for healthy broiler meat has increased recently. Trends in the use of conventional antibiotic growth promoters (AGPs) in broiler diet have become obsolete due to their negative effects to generate resistant pathogenic bacteria and their residual presence in broiler products (Bahar and Ren 2013; Leeson and Summers 2009). Accordingly, there is a need to substitute AGP with other compounds particularly those originated or derived from nature like antimicrobial peptides (Gadde *et al.* 2017; Xiao *et al.* 2015; Wang *et al.* 2016). Antimicrobial peptide (AMP) is composed of 4 to 99 amino acids (mostly cationic) that can act as an antifungal, antiviral, antibacterial (i.e bacteriocide and bacteriostatic), immunomodulatory, anticancer, antitumor, and antioxidant agent (Bahar and Ren 2013; Ikeda 2001; Li *et al.* 2012; Park and Yoe 2017a, 2017b; Wu *et al.* 2018; Yi *et al.* 2014; Zhao *et al.* 2013). AMP substances can be isolated from animal tissues (e.g lactoferrin, colostrum, swine antibacterial peptide, and lysozyme), recombinant product (e.g cecropin AD-asparagin and microcin J25), plants (e.g thionin and potamin), insects (e.g defensin-like peptides and diptericin), microbes (e.g gramicidin and nisin) and amphibians (e.g magainin) (Bahar and Ren 2013; Ikeda 2001; Kim *et al.* 2005; Li *et al.* 2017; Park and Yoe 2017b; Wang *et al.* 2020; Zhao *et al.* 2013). The use of AMP as an alternative to substitute conventional AGPs has advantages such as high stability against digestive enzyme degradation i.e cysteine-rich peptide (Silva *et al.* 2000). Also, it tends not to cause resistance effects (due to the β-sheet structure) and has a broad spectrum against various types of pathogens (Bradshaw 2003; Yi *et al.* 2014).

Based on *in vitro* studies, the AMP substance, such a defensin, can inhibit gram-positive bacteria (e.g *Bacillus subtilis* and *Staphylococcus aureus*), *Escherichia coli,*

and other types of fungi (Li *et al.* 2012; Wang *et al.* 2016). In addition, *in vitro* studies also reported the reduction of oxidative stress as the effect of AMP addition (Ikeda 2001, Wang *et al*. 2019). Furthermore, *in vivo* study reported the success of AMP to increase productivity through the improvement of the immune response and small intestine ecosystem in the broiler (Choi *et al.* 2013a, 2013b; Wang *et al.* 2020). The addition of AMP also shows a positive response to the antibody titer (Bai *et al.* 2019). Also, Gong *et al.* (2016) report that lysozyme administration in broilers had no effect on aerobic bacteria, coliforms, and *Clostridium perfringens*. Therefore, this study was conducted to assess the effects of AMP addition on the number of bacteria, immune responses, and antioxidant activity of broiler by integrating data from previous published reports.

MATERIALS AND METHODS

Database Development

A database was developed based on literatures that reported effects of AMP addition on the number of bacteria, immune responses, and antioxidant activity of broiler. The literatures were found in Science Direct and Google Scholar, by using various keywords such as "antimicrobial peptide", "bacterial number", "immune response", "antioxidant activities" and or "broiler". A total of 43 journal articles with digital object identifiers were found. After title and abstract suitability evaluation, 29 articles were entered in the database. The evaluation criteria used were: (1) the article was published in English, (2) the AMP level was determined, and (3) the *in vivo* experiment used a fast-growing broiler. If an article consisted of two or more experiments, the experiments were individually encoded. In total there were 36

77 experiments used for meta-analysis that comprised of 111 data points as depicted in
78 Table 1. This meta-analysis study followed the preferred reporting items for systematic
79 review and meta-analysis protocols (PRISMA-P) (Shamseer *et al.* 2015).

80 The addition levels of AMP were varied, in a range of 0 (control) to 600 mg kg^{-1}
81 of diet. The used AMP was derived from animal tissue purification (e.g., swine
82 antibacterial peptides, lactoferrin and bee venom), recombinant products (i.e., microcin
83 J25, AMP-A3 and AMP-P5), and plant-based protein extraction (i.e., bioactive peptides
84 from canola, sesame and soybean). Broilers were maintained in two phases: starter
85 (ranged between 1-21 days) and finisher (ranged between 22-42 days). Broiler strains
86 used in the meta-analysis were varied, namely Arbor Acres, Cobb 500, Lingnan,
87 Lohmann, Hubbard, and ROSS 308.

88 The assessed variables were the number of bacteria (e.g *Clostridium* spp.,
89 *Escherichia coli*, coliform, lactic acid bacteria (LAB), and total aerobic bacteria
90 (TAB)), immune responses (e.g immunoglobulin A (IgA), immunoglobulin M (IgM),
91 cluster of differentiation 3 (CD3), cluster of differentiation 4 (CD4), antibody titer,
92 bursal index, spleen index, thymus index), and antioxidant activity (e.g total superoxide
93 dismutase (TSOD), total antioxidant activity (TAA), and superoxide dismutase activity
94 (SOD activity)). Data on growth performance, carcass characteristics and small
95 intestinal morphology were excluded since they were presented in a separated paper and
96 submitted elsewhere (Sholikin *et al.* 2020).

97

98 **Data Analysis**

99 Data analysis was performed in R software version 3.6.3 with additional
100 packages such as "nlme" and "tidyverse" (Bates *et al.* 2015; Pinheiro *et al.* 2020; R

Core Team 2020). Linear mixed models (LMM) methodology was performed for the present meta-analysis. The addition level of AMP was fixed effects, while the experiment was random effects (Gałecki and Burzykowski 2013; Sauvant *et al.* 2008; St-Pierre 2001). The mathematical model follows the following equation.

$$Y_{ij} = \beta_0 + \beta_1 Level_{ij} + Experiment_i + Experiment_i Level_{ij} + e_{ij} \quad (1)$$

$$Y_{ij} = \beta_0 + \beta_1 Level_{ij} + \beta_2 Level_{ij}^2 + Experiment_i + Experiment_i Level_{ij} + e_{ij} \quad (2)$$

Where (1) linear mixed model of the 1st order 1, (2) linear mixed model of the 2nd order, Y_{ij} = dependent variable, β_0 = overall intercept across all studies (fixed effect), β_1 = linear regression coefficient of Y on Level (fixed effect), β_2 = quadratic regression coefficient of Y on Level (fixed effect), $Level_{ij}$ = value of the continuous predictor variable (AMP addition level), $Experiment_i$ = random effect of study i, $Experiment_i Level_{ij}$ = random effect of study i on the regression coefficient of Y on Level in study i, e_{ij} = the unexplained residual error. The p-value, root mean square error (RMSE), and akaike information criterion (AIC) were used to evaluate the suitability of statistical models (Gałecki and Burzykowski 2013; Chai *et al.* 2014). If the p-value was less than or equal to 0.05, the result was significant. In addition, there was a tendency to be significant if only the p-value ranged between 0.05 and 0.1.

RESULTS

The effect of AMP addition level on the number of bacteria is shown in Table 2. In ileum, the number of bacteria (coliform and TAB) linearly declined ($P < 0.05$) with the increasing AMP level in starter broiler. Similarly, *Escherichia coli* population linearly decreased ($p < 0.05$) due to the AMP addition for finisher broiler, while the

122 TAB tended to decrease linearly ($p < 0.1$). In caecum of starter broiler, there was a linear
123 decrease of bacterial number, such as *Clostridium* spp., coliform, *Escherichia coli*, and
124 LAB ($p < 0.05$) following the AMP addition. Meanwhile, the TAB tended to have a
125 linear increase in finisher broiler ($p < 0.1$). In excreta of starter broiler, the number of
126 *Clostridium* spp. tended to decline linearly ($p < 0.1$). Other bacteria species in small
127 intestine were not affected by the AMP addition.

128 The AMP addition possessed a linear pattern on immune response ($p < 0.05$) and
129 antioxidant activity ($p < 0.1$) of broiler (Table 3). In starter phase, AMP addition linearly
130 increased ($p < 0.05$) ND antibody titers and lymphoid organs (i.e., bursal index, spleen
131 index, and thymus index). Similarly, immunoglobulin and complement (IgM; CD4),
132 ND antibody titer, and spleen organs of finisher broiler increased in linear pattern due to
133 AMP addition ($p < 0.05$; Table 3), whereas IgA and CD3 were not affected. The effect
134 of AMP addition tended ($p < 0.1$) to linearly elevate SOD activity, while TAA was not
135 influenced in finisher broiler. The addition of AMP did not affect TSOD in starter
136 broiler.

137 Previous study by Sholikin *et al*. (2020) showed that optimal AMP levels based
138 on feed conversion ratio variables were 337, 359, and 371 mg kg^{-1}, in the starter,
139 finisher, and total phases, respectively. The reduction of total *Clostridium* spp. was
140 following equation (3). This was reduced by 8.85% or from 7.24 to 6.60 log10 cfu g^{-1}.
141 The normal rate of *Clostridium* spp. ranged from 7.15 up to 7.27 log10 cfu g^{-1} at ileum
142 broiler starter (Choi *et al*. 2013b; Chowdhury *et al*. 2018). Based on equation (4), IgM
143 increased for about 49.33% from 0.58 to 0.87 g L^{-1}. The IgM under normal conditions
144 by Ma *et al*. (2019) is 0.50 g L^{-1}. Based on equation (5), SOD activity increased from

9.35 up to 21.92% inhibition. Karimzadeh *et al*. (2017b) reported that normal broiler SOD activity was 11.40% inhibition.

$$Y_{Clostridiumspp.} = 7.24 - 0.00191\, X_{level};\ (p = 0.007) \quad (3)$$

$$Y_{IgM} = 0.58 + 0.000797\, X_{level};\ (p = 0.037) \quad (4)$$

$$Y_{SODactivity} = 9.35 + 0.0351\, X_{level};\ (p = 0.01) \quad (5)$$

Where (3) *Clostridium* spp. regression equation based on Table 2 row 10, (4) IgM regression equation based on Table 3 row 2, (5) SOD activity regression equation based on Table 3 row 17, Y = dependent variable (variable), X = independent variable (level of AMP).

DISCUSSION

Effect of AMP Addition on Bacteria Population in Small Intestine of Broiler

In general, AMP addition is able to reduce the number of pathogenic bacteria in small intestine of broiler both in starter and finisher phases. Pathogenic bacteria in small intestine may cause a variety of negative effects, especially tissue damage and also the production of toxic compounds. The accumulation of toxic compounds leads to the emergence of various types of metabolic diseases and may reduce growth performance, nutrient digestibility, and immune response. With regard to the effect of AMP on pathogenic bacteria, present finding highlights the reduction of number of *Clostridium* spp. *Clostridium* spp. is a gram-positive bacterium that causes botulism (Chalk *et al.* 2019; Johnson 2019). The percentage of *Clostridium* spp. found in ileum and caecum of broiler were 9.69% and 39.26% of total bacteria, respectively (Lu *et al.* 2003). Choi *et al.* (2013a) reported the decline of *Clostridium* spp. in excreta due to AMP-A3 addition

(starter and finisher phase). The decline of *Clostridium* spp. is possibly due to the ability of AMP in form of cecropin-A-maganin-2 (CAMA) to inhibit or even kill gram-positive bacteria (Vizioli *et al.* 2000). CAMA as composed of an amphypatic terminal base in CA and N-terminal (hydrophobic region) base in MA that both terminals were effective in damaging bacterial cell membranes (Park and Yoe 2017a; Xiao *et al.* 2015; Yue *et al.* 2020; Zhang *et al.* 2017).

Escherichia coli and TAB are categorized as coliform group bacteria (Malcolm 1938). Coliform possess several characteristics such as gram negative, lactose base energy source, and aerobic or anaerobic facultative (Malcolm 1938). Bacteria in this group were able to produce various types of toxic such as indole, skatole, and ethionine that may trigger cancer and cause diarrhea (Anabrees *et al.* 2013; Girard and Bee 2020). Present study confirms the reduction of coliform bacteria number like *Escherichia coli* in ileum and caecum due to AMP addition. This finding was in accordance with previous studies that showed the reduction of coliform bacteria in ileum after the addition of AMP-P3, lysozyme, and sesame meal bioactive peptide (Choi *et al*. 2013b; Gong *et al*. 2017; Salavati *et al*. 2019). Some types of AMP such as cecropin (isolated from *Hermetia illucens*) and lysozyme were also effective to inhibit gram negative bacteria like *Escherichia coli* (Pellegrini *et al.* 1992; Park and Yoe 2017a). Lysozyme was able to hydrolyze cell walls of both gram-positive and gram-negative bacteria that composed of peptidoglycan (Ragland and Criss 2017). The number of TAB decreased in small intestine and also feces due to the addition of AMP in form of AMP-A3, AMP-P5, cecropin, and recombinant plectacin (Choi *et al.* 2013b, 2013a; Ma *et al.* 2019; Wen and He 2012).

188 In contrast to the present finding, Salavati *et al.* (2019) reported the increase of
189 LAB number as due to lysozyme. Those different findings might be related to the
190 diversity of interactions of AMP against various types of LAB. For instance, lysozyme
191 was reported to have inhibitory activity against several types of LAB like *Lactobacillus*
192 *brevis* (Tribst *et al.* 2008). Lüders *et al.* (2003) reported that LAB such as *Lactobacillus*
193 *curvatus* LTH1174 and *Pediococcus acidilactici* LMG 2351 were capable of producing
194 AMPs Curvacin A and Pediocin PA-1.
195 The reduction of *Clostridium perfringens* population for about 10.9% increased
196 the population of LAB in ileum for about 2.3% (Askelson *et al*. 2018). Based on 16S
197 rDNA sequences, the number of *Lactobacillus* spp. in ileum of broiler was around 67%
198 of total bacteria (Lu *et al.* 2003). *Lactobacillus* spp. could adhere to small intestine
199 walls and also capable of producing organic acids such as short chain fatty acids (e.g.,
200 butyric, propionic, and acetic) and also lactic acid (Rowland *et al.* 2018). These organic
201 acids reduce pH in small intestine and provide energy that available for epithelial cells
202 (Krajmalnik-Brown *et al.* 2012; Shang *et al.* 2018). Energy availability increases cell
203 metabolism so that small intestinal morphology could be maintained. In addition, LAB
204 and *Bacillus subtilis* were reported to increase gene expression from mucin that was
205 useful for maintaining mucosa thickness (Aliakbarpour *et al.* 2012).
206
207 **Effect of AMP Addition on Immune Response and Antioxidant Activity of Broiler**
208 Generally, AMP addition positively affects the broiler immune response such as
209 immunoglobulin, complement, ND antibody titer and lymphoid organs.
210 Immunoglobulin is the product of B cells (humoral immunity) used to fight antigens
211 (Schat *et al*. 2013). IgA serves an important role in mucosal immunity (in parts of

212 body's secretory organs, respiratory tract, digestive tract, and skin surface) to prevent
213 the attachment of bacteria and viruses to the mucous membrane (Bonner *et al.* 2009;
214 Fagarasan and Honjo 2003; Macpherson and Slack 2007; Schat *et al.* 2013).
215 Meanwhile, IgM has a role as a binder of bacteria that attached to the mucosa (Jazayeri
216 *et al.* 2019; Murguia-Favela *et al.* 2017; Sharma 2017). Complement is a part of cellular
217 immunity and has an important role on T lymphocytes. The function of CD3 is to
218 activate cytotoxic T cells and T helper cells while CD4 is a receptor of T helper cells
219 that act as a marker (communicating with antigen-presenting cells) (Schat *et al.* 2013).
220 Similar to Bai *et al.* (2019) finding, the lymphoid organ index was reported to increase
221 in this study. The thymus is the site of differentiation of T lymphocytes, while the bursa
222 of fabricius is a site of maturation of B lymphocytes (Schat *et al.* 2013). In line with an
223 improvement in serum immunoglobulin and complement variables, broilers challenged
224 by the *Newcastle disease* virus and given AMP could increase their antibody titers in
225 both starter and finisher phases. Similar findings by Bai *et al.* (2019) who used cecropin
226 and seaweed powder to increase antibody titers. The increase of IgM, CD4 cell,
227 lymphoid organ index, and antibody titer has a positive effect on the immune status of
228 broilers. AMP increased innate and adaptive immunity by improving proinflammatory
229 and anti-inflammatory modulation, chemotaxis activity, and direct effects on adaptive
230 immunity (Wang *et al.* 2016). AMP increased the number of T cells and their
231 proliferation product in blood peripherals, and also increased IgG, IgM, and IgA in pigs
232 (Ren *et al.* 2015; Yuan *et al.* 2015).

233 Antioxidant activity of broiler could be assessed based on its SOD activity
234 status. Similar result to the present finding, Karimzadeh *et al.* (2017b) reported the
235 increase of SOD activity in broilers at 42 days by AMP addition in the form of

236 recombinant plectacin. SOD is an enzyme for neutralizing the activity of free radicals
237 such as peroxide and superperoxide (Corpas *et al.* 2006). The proline or arginine-rich
238 AMP (PR-39) proved to inhibit the activity of nicotinamide adenine dinucleotide
239 phosphate oxidase (NADPH oxidase) from polymorphonuclear leukocytes by blocking
240 the assembly of these enzymes (Ikeda 2001). The NADPH oxidase itself is the main
241 source of superperoxide. Ability of AMP to suppress free radicals was reported through
242 two main mechanisms, i.e., increasing SOD activity and catalyzing enzymes, and
243 damaging the integrity of NADPH oxidase that is influenced by the activity of N-
244 terminal groups and carboxylic acid groups (Ikeda 2001; Xiao *et al*. 2015).

CONCLUSION

The present meta-analysis revealed the effect of AMP addition in form of the decline not only the number of *Clostridium* spp. at caecum and excreta in starter broiler but also the number of *Escherichia coli* at ileum in finisher broiler and at caecum in starter broiler. Moreover, the number of coliform at ileum and caecum in starter broiler, and TAB at ileum in starter and finisher broiler were decreased as the effect of the addition of AMP. The immune response and antioxidant activity of broiler could also be improved as indicated by the positive responses of serum immunoglobulin M and cluster of differentiation 4, antibody titer, index of lymphoid organs, and SOD activity.

CONFLICT OF INTEREST

We declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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568 Table 1. Literature included in the meta-analysis of antimicrobial peptide addition (mg kg^{-1} of diet) on bacterial population in small intestine and
569 immune response of broiler

Exp.	Antimicrobial Peptides	Sources	Level	Broiler	Sex	Starter	Finisher	Total	References
1.	Swine antibacterial peptides	Swine intestine	0-200	Arbor Acres	Male	1-21	22-42	1-42	Bao *et al.* (2009)
2.	Swine antibacterial peptides	Swine intestine	0-30	Arbor Acres	Male	1-21	22-42	1-42	
3.	Refined potato protein	*Solanum tuberosum* L.	0-600	ROSS 308	Male	1-21	22-42	1-42	Ohh *et al.* (2009)
4.	AMP-A3	*Helicobacter pylori*	0-90	ROSS 308	-	1-21	22-35	1-35	Choi *et al.* (2013a)
5.	AMP-P5	Analog of Cecropin	0-60	ROSS 308	-	1-21	22-35	1-35	Choi *et al.* (2013b)
6.	Lysozyme	-	0-120	ROSS 308	-	1-21	22-35	1-35	Abdel-Latif *et al.* (2017)
7.	Recombinant plectasin	*Saprophytic ascomycete*	0-200	Arbor Acres	Male	1-21	22-42	1-42	Ma *et al.* (2019)
8.	Camel lactoferrin chimera	-	0-20	Cobb 500	Male	1-10	11-24	1-24	Daneshmand *et al.* (2019a)
9.	Lysozyme	Egg white	0-40	ROSS 308	Male	14-28	29-33	14-33	Torki *et al.* (2018)
10.	Peptide	-	0-250	-	-	1-10	11-28	1-42	Karimzadeh *et al.* (2017a)
11.	Sublancin	*Bacillus subtilis*	0-11.52	Arbor Acres	-	1-21	22-28	1-28	Wang *et al.* (2015)

Exp.	Antimicrobial Peptides	Sources	Level	Broiler	Sex	Starter	Finisher	Total	References
12.	Lysozyme	Egg white	0-100	ROSS 308	Male	1-24	25-35	1-35	Gong *et al.* (2017)
13.	Swine antibacterial peptides	Swine intestine	0-0.1	Lohmann	-	-	-	1-42	Wang *et al.* (2009)
14.	Cecropin AD-asparagin	*Hyalophora cecropia*	0-8	Lingnan	Male	14-28	29-42	14-42	Wen and He (2012)
15.	Bee venom	*Apis mellifera* L.	0-1	Arbor Acres	-	1-28	-	1-28	Han *et al.* (2010)
16.	Glucagon-like peptide 2	-	0-0.33	Arbor Acres	-	1-21	-	1-21	Hu *et al.* (2010)
17.	Glucagon-like peptide 2	-	0-0.33	Arbor Acres	-	1-21	-	1-21	
18.	Lysozyme	-	0-200	Cobb 500	Male	1-28	-	1-28	Zhang *et al.* (2010)
19.	Lysozyme	-	0-200	Cobb 500	Male	1-28	-	1-28	
20.	Bee venom	*Apis mellifera*	0-0.5	ROSS 308	Male	1-21	-	1-35	Kim *et al.* (2018)
21.	Sesame bioactive peptides	*Sesamum indicum*	0-150	ROSS 308	-	1-24	25-35	1-35	Salavati *et al.* (2019)
22.	Soybean bioactive peptides	*Glycine max*	0-200	Arbor Acres	-	1-28	29-49	1-49	Jiang *et al.* (2009)
23.	Lysozyme	-	0-40	Arbor Acres	Male	1-14	15-28	1-28	Liu *et al.* (2010)
24.	Lysozyme	-	0-40	Arbor Acres	Male	1-14	15-28	1-28	Liu *et al.* (2010)

Exp.	Antimicrobial Peptides	Sources	Level	Broiler	Sex	Starter	Finisher	Total	References
25.	Canola bioactive peptides	*Brassica* spp.	0-250	ROSS 308	Male	1-28	29-42	1-42	Karimzadeh *et al.* (2016)
26.	Canola bioactive peptides	*Brassica* spp.	0-250	ROSS 308	Male	1-28	29-42	1-42	Karimzadeh *et al.* (2017b)
27.	Cecropin	*Bombyx mori*	0-600	Arbor Acres	Mix	1-21	22-42	1-42	Bai *et al.* (2019)
28.	Cecropin	*Bombyx mori*	0-600	Arbor Acres	Mix	1-21	22-42	1-42	
29.	Cecropin	*Bombyx mori*	0-600	Arbor Acres	Mix	1-21	22-42	1-42	
30.	Cecropin	*Bombyx mori*	0-300	Arbor Acres	Mix	1-21	22-42	1-42	
31.	Camel lactoferrin 36	-	0-20	Cobb 500	Male	1-22	-	1-22	Daneshmand *et al.* (2019b)
32.	Bovine lactoferrin	-	0-500	Cobb 500	Male	1-24	25-32	1-32	Geier *et al.* (2011)
33.	Bee venom	*Apis mellifera carnica*	0-1.5	ROSS 308	Mix	1-21	22-42	1-42	Ali and Mohanny (2014)
34.	Bovine lactoferrin	-	0-520	Cobb 500	-	8-28	29-42	8-42	Aguirre *et al.* (2015)
35.	Lactoferrin	-	0-250	Hubbard	Mix	-	-	1-42	Enany *et al.* (2017)
36.	Microcin J25	-	0-1	Arbor Acres	Male	1-21	22-42	1-42	Wang *et al.* (2020)

570 Note: AMP, Antimicrobial peptide; Exp, Number of experiment

571 Table 2. The regression equation of the AMP (mg kg^{-1} of diet) on the number of bacteria (log10 cfu $gram^{-1}$) of broiler

No.	Response variable	Model	N	Variable estimates				Model estimates			
				Int.	SE Int.	Slope	SE Slope	p-value	RMSE	AIC[1)]	Trend
Ileum microbes, Starter											
1.	*Clostridium* spp.	L	16	4.2	0.962	-0.004	0.0028	0.198	1.02	49.8	Neg.
2.	Coliform	L	10	4.86	0.663	-0.00489	0.0004	<0.001	0.85	11.1	Neg.
3.	*Escherichia coli*	L	6	4.24	0.269	-0.000987	0.0024	0.715	0.79	9.52	Neg.
4.	LAB	L	6	6.72	0.398	0.00181	0.0094	0.865	1.08	20.1	Pos.
5.	TAB	L	11	7.73	0.45	-0.00416	0.0011	0.011	0.87	17.7	Neg.
Ileum microbes, Finisher											
6.	Coliform	L	6	5.11	0.159	-0.000265	0.0002	0.184	0.88	-2.59	Neg.
7.	*Escherichia coli*	L	8	5.24	0.66	-0.00354	0.0009	0.015	0.97	10.4	Neg.
8.	LAB	L	8	7.49	0.255	-0.000086	0.0034	0.981	1.18	17.8	Neg.
9.	TAB	L	16	7.25	0.656	-0.00293	0.0014	0.059	1.07	42.7	Neg.
Caecum microbes, Starter											
10.	*Clostridium* spp.	L	6	7.24	0.0293	-0.00191	0.0003	0.007	0.85	-18.8	Neg.
11.	Coliform	L	6	5.6	0.791	-0.0038	0.0011	0.038	0.82	5.35	Neg.
12.	*Escherichia coli*	L	18	6.96	0.482	-0.0012	0.0005	0.025	1.26	44	Neg.
13.	LAB	L	15	7.05	0.0786	-0.00111	0.0002	0.002	1.38	3.33	Neg.
14.	TAB	L	13	8.25	0.49	-0.00131	0.0008	0.131	1.07	13.4	Neg.
Caecum microbes, Finisher											

No.	Response variable	Model	N	Variable estimates				Model estimates			
				Int.	SE Int.	Slope	SE Slope	p-value	RMSE	AIC[1)]	Trend
15.	Coliform	L	6	3.62	0.818	-0.000808	0.0011	0.500	0.9	19.7	Neg.
16.	*Escherichia coli*	L	18	7.14	0.667	0.000421	0.0003	0.151	0.91	37.2	Pos.
17.	LAB	L	15	7.57	0.282	0.000403	0.0002	0.083	1.08	15.9	Pos.
18.	TAB	L	12	7.77	0.462	-0.00103	0.0010	0.314	1.24	29.5	Neg.
	Excreta microbes, Starter										
19.	*Clostridium* spp.	L	10	7.22	0.307	-0.00472	0.0021	0.070	0.88	14.4	Neg.
20.	Coliform	L	10	6.7	0.317	-0.00351	0.0048	0.489	1.17	24.1	Neg.
21.	TAB	L	14	7.6	0.747	-0.000238	0.0008	0.772	1.39	33.9	Neg.
	Excreta microbes, Finisher										
22.	*Clostridium* spp.	L	10	7.72	0.334	-0.00195	0.0012	0.159	1.14	7.1	Neg.
23.	Coliform	L	14	6.296	0.422	-0.000854	0.0009	0.363	1.35	31.8	Neg.
24.	TAB	L	14	7.839	0.522	-0.000371	0.0007	0.599	1.36	27.9	Neg.

572 Note: AIC, Akaike information criterion; Int., Intercept; LAB, Lactic acid bacteria; L, Linear; N, Number of data; Neg., Negative; Pos. Positive;
573 RMSE, Root mean square error; SE, Standard error; TAB, Total aerobic bacteria; [1)]AIC is an estimator of the relative quality of statistical models for a
574 given set of data.

575 Table 3. The regression equation of the AMP (mg kg^{-1} of diet) on immune response and antioxidant activities of broiler

No.	Response variable	Unit	Model	N	Variable estimates				Model estimates			
					Int.	SE Int.	Slope	SE Slope	p-value	RMSE	AIC[1)]	Trend
Serum Immunoglobulin and complement, Finisher												
1.	IgA	g/L	L	8	0.657	0.38	6.00E-05	0.0001	0.689	1.06	-12	Pos.
2.	IgM	g/L	L	8	0.58	0.13	0.000797	0.0003	0.037	0.95	-8.15	Pos.
3.	CD3	g/L	L	6	2.49	0.728	0.000775	0.0005	0.204	0.83	11.3	Pos.
4.	CD4	g/L	L	6	0.886	0.639	0.000698	0.0002	0.032	0.83	3.07	Pos.
Newcastle disease antibody titer, Starter[2)]												
5.	Antibody titer	^{2}log(N)	L	13	2.71	0.799	0.00145	0.0003	0.002	1.13	29.4	Pos.
6.	Antibody titer	%	L	11	30.4	1.29	0.0114	0.0028	0.007	1.2	57.9	Pos.
Newcastle disease antibody titer, Finisher[2)]												
7.	Antibody titer	^{2}log(N)	L	17	6.2	0.791	0.00122	0.0006	0.069	1.15	51.4	Pos.
8.	Antibody titer	%	L	11	33.6	1.5	0.0105	0.0033	0.019	1.23	61.3	Pos.
Lymphoid organ index, Starter												
9.	Bursal index		L	11	2.49	0.033	0.000318	0.0001	0.007	1.27	-21.9	Pos.
10.	Spleen index		L	11	0.94	0.0138	0.000151	0.0000	0.004	1.3	-40.9	Pos.
11.	Thymus index		L	11	4.76	0.233	0.00172	0.0005	0.019	1.22	20.8	Pos.
Lymphoid organ index, Finisher												
12.	Bursal index		L	11	1.6	0.0717	0.000509	0.0002	0.032	1.34	-4.31	Pos.

No.	Response variable	Unit	Model	N	Variable estimates				Model estimates			
					Int.	SE Int.	Slope	SE Slope	p-value	RMSE	AIC[1)]	Trend
13.	Spleen index		L	11	1.26	0.0145	0.00014	0.0000	0.006	1.27	-40.2	Pos.
14.	Thymus index		L	11	5.07	0.0689	0.000721	0.0002	0.006	1.26	-5.37	Pos.
	Antioxidant activity, Starter											
15.	Total superoxide dismutase	U/mg	L	6	43.8	15.8	0.0107	0.0272	0.720	0.84	48	Pos.
	Antioxidant activity, Finisher											
16.	Total antioxidant activity	U/mg	L	8	1.81	0.53	0.000782	0.0012	0.538	0.94	8.57	Pos.
17.	Superoxide dismutase	% inhibition	L	5	9.35	2.47	0.0351	0.0150	0.101	1	30.2	Pos.

576 Note: AIC, Akaike information criterion; CD3, Cluster of differentiation 3; CD4, Cluster of differentiation 4; IgA, Immunoglobulin A; IgM,
577 Immunoglobulin M; Int., Intercept; L, Linear; N, Number of data; Neg., Negative; Pos. Positive; RMSE, Root mean square error; SE, standard error;
578 [1)]AIC is an estimator of the relative quality of statistical models for a given set of data; [2)]Antibody titer tested using *Newcastle disease* virus.