Influence of humic acid supplemented diets on intestinal microbiome and laying performance of egg-type chicken

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Abstract

Increasing prevalence of antibiotic resistance due to its indiscriminate use in poultry production is an emerging public health issue. This has prompted research on viable alternatives that will improve production performance of birds. This study was designed to evaluate the effect of humic acid supplemented diets on production performance and microbial load in laying hens. Fifty-four 20-week old Isa brown hens were randomly distributed into three dietary treatments consisted of six replicates with three birds per replicate in a completely randomized design. Treatment 1 was the basal diet (control diet) with no humic acid supplementation while treatments 2 and 3 had the basal diet supplemented with 0.2% humic acid and 0.4% humic acid, respectively evaluated in a 42-day feeding trial. Performance parameters were evaluated. On day 42, the egg quality attributes were measured. Birds were slaughtered and digesta samples from the ileum were collected for microbial load count. Data obtained were analyzed using descriptive statistics and ANOVA at $\alpha 0.05$. Birds fed 0.4% humic acid supplemented diet had the highest final weight (1721g/bird) and weight gain (97.83g/bird). A decrease in weight was observed in birds fed with 0% humic acid (-168.17g/bird) and 0.2% humic acid (-24.17g/bird) supplemented diets. Higher ($P < 0.05$) yolk height and yolk width were recorded in supplemented diets as compared with the control diet. Lowest yolk colour (2.33) was found in the control diet while highest (4.16) in 0.4% humic acid diet. Similarly, highest ($P < 0.05$) Haugh unit (78.09) and shell weight (7.83g) were recorded in 0.4% humic acid supplemented diet. Least total heterotrophic count (1.10×10^3 CFU/ml) was recorded in 0.2% humic acid diet while highest total Lactobacilli count (0.23×10^3 CFU/ml) observed in 0.4% humic acid diet. In conclusion, humic acid supplementation up to 0.4% can serve as a viable replacement to antibiotic growth promoter for improved gut health and performance of laying birds.

Keywords: Laying hens, Humate, Production performance, Microbiota, Egg quality attributes

Influence des régimes alimentaires enrichis en acide humique sur les performances de ponte et le microbiome intestinal des poules de type œuf

Résumé

La prévalence croissante de la résistance aux antibiotiques en raison de son utilisation inconsidérée dans la production avicole est un problème de santé publique émergent. Cela a suscité des alternatives viables qui stimuleront la réponse immunitaire des oiseaux et amélioreront les performances de production. Cette étude a été conçue pour évaluer l'effet des régimes alimentaires enrichis en acide humique sur les performances de production et la charge microbienne chez les poules pondeuses. Cinquante-quatre poules brunes Isa âgées de 20 semaines ont été réparties au hasard dans trois traitements diététiques composés de six répétitions avec trois oiseaux par répétition dans un plan complètement randomisé. Le traitement 1 était le régime de base (régime témoin) sans supplémentation en acide humique,

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tandis que les traitements 2 et 3 avaient le régime de base complété avec 0,2 % d'acide humique et 0,4 % d'acide humique, évalués respectivement dans un essai d'alimentation de 42 jours. Les paramètres de performance ont été évalués. Au jour 42, les attributs de qualité des œufs ont été mesurés. Les oiseaux ont été abattus et des échantillons de digesta de l'iléon ont été collectés pour le décompte de la charge microbienne. Les données obtenues ont été analysées en utilisant des statistiques descriptives et une ANOVA à α 0,05. Les résultats ont montré que les oiseaux nourris avec une alimentation enrichie en acide humique à 0,4% avaient le poids final (1721 g/oiseau) et le gain de poids (97,83 g/oiseau) les plus élevés. Une diminution du poids a été observée chez les oiseaux nourris avec des régimes supplémentés à 0 % d'acide humique (-168,17 g/oiseau) et 0,2 % d'acide humique (-24,17 g/oiseau). Une hauteur et une largeur du jaune plus élevées ($P < 0,05$) ont été enregistrées dans les régimes supplémentés par rapport au régime témoin. Une couleur plus faible du jaune (2,33) a été trouvée dans le régime témoin alors qu'elle était plus élevée (4,16) dans le régime contenant 0,4 % d'acide humique. De même, une unité Haugh (78,09) et un poids de coquille (7,83 g) plus élevés ($P < 0,05$) ont été enregistrés dans un régime enrichi en acide humique à 0,4 %. Le nombre total d'hétérotrophes le plus faible ($1,10 \times 10^2$ cfu/ml) a été enregistré dans un régime contenant 0,2 % d'acide humique, tandis que le nombre total de lactobacilles le plus élevé ($0,23 \times 10^2$ cfu/ml) a été observé dans un régime contenant 0,4 % d'acide humique. En conclusion, une supplémentation en acide humique jusqu'à 0,4% peut servir de remplacement viable au promoteur de croissance antibiotique pour améliorer la santé intestinale et les performances des oiseaux pondeurs.

Mots clés : Poules pondeuses, humate, performance de production, microbiote, attributs de qualité des œufs

Introduction

High population growth and growing income lead to increasing demand for poultry products in Nigeria. Thus, intensification of poultry production to meet the demand of ever-increasing human population becomes necessary. As a result of this, antibiotics are often administered to prevent the incidence of diseases, improve nutrient utilization and growth performance of birds (Mathur and Singh, 2005; Stanton, 2013). However, indiscriminate use of antibiotics has undesirable effect on birds, such as residue in the tissues and development of resistance genes and transference of such to human microbiota (Hughes and Heritage, 2004). Therefore, the use of antibiotics as growth promoters has been banned in many countries especially in the European Union (EU, 2006; Castanon, 2007) and followed by a similar legislation by issued in Nigeria by

the National Agency for Food and Drug Administration and Control (NAFDAC, 2018). Therefore, poultry researchers and nutritionists are searching for viable alternative feed additives that can conveniently replace antibiotics growth promoters in poultry feed without eliciting negative impact on the food chain (Diarra and Malouin, 2014). There are a number of non-therapeutic alternatives that can serve as alternatives to antibiotic growth promoters in poultry nutrition. Among these are: probiotics and prebiotics (Patterson and Burkholder, 2003; Jung *et al.*, 2008), organic acids (Diebold and Eidelsburger, 2006; Agboola *et al.*, 2015), enzymes (Bedford, 2000), herbs and plant extracts (Demir *et al.*, 2005; Omidiwura *et al.*, 2020) etc. The inclusion of organic acids, such as humic acid, as a feed additive in broiler diets can have multiple health and nutritional benefits (Ozturk *et al.*, 2012; Ragaa and Korany, 2016).

Humic acid is an organic substance derived from the decomposition of organic matter and having a long molecular chain high in its molecular weight (MacCarthy, 2001). Humic acid has been reported to enhance growth through modifying partitioning of nutrient metabolism in broiler chickens (Abdel-Mageed, 2012; Taklimi *et al.*, 2012) and also has the ability to alter the intestinal microflora by increasing the counts of beneficial bacteria (Schepetkin *et al.*, 2003). They have been used as an antidiarrheal, analgesic, immunostimulatory and antimicrobial agent in veterinary practices in Europe (EMEA, 1999). It has also been proposed that humic acid plays an effective role in minimizing health problems and potential losses in laying hens (Yoruk *et al.*, 2004; Kucukersan *et al.*, 2005). Against this background, the study was designed to evaluate the effect of humic acid supplementation on laying performance, egg quality attributes and microbial load in laying hens.

Materials and methods

Experimental site

This experiment was carried out at the Poultry Unit, Teaching and Research Farm, University of Ibadan, Oyo State, Nigeria.

Experimental diets and management of birds

Fifty-four 20-week old Isa brown hens from the Poultry Unit, Teaching and Research Farm, University of Ibadan were used for this experiment. The birds were randomly distributed into three dietary treatments sorted by body weight with six replicates comprised of three hens each in a completely randomized design. The birds were housed in a battery cage in a semi-controlled environment. The birds had access to 100g of experimental diet per bird per day and water was supplied *ad libitum* during the study period which lasted for six weeks. The basal diet was a standard

commercial bagged layers' mash containing: Crude protein-16.5%, Calcium-3.6%, Available phosphorus- 0.45% and Metabolizable energy- 2,500 Kcal/kg in accordance with NRC (1994) guidelines. Treatment 1 was the control diet (basal diet), not supplemented with humic acid. Treatment 2 had basal diet + 0.2% humic acid while Treatment 3 was a basal diet + 0.4% humic acid. Respective humic acid concentration was added over the top of the compounded feed.

Data collection

Feed intake

Each replicate was supplied 100g of feed in the morning and 200g of feed in the afternoon making a total of 300g of feed per replicate (3 birds) per day. Feed leftover was measured at the end of each week. Weekly feed intake was calculated by subtracting the leftover feed from the feed supplied. Daily feed intake was calculated by dividing the weekly feed intake by 7.

Weight gain

The birds were weighed weekly with the use of a sensitive weighing scale to determine the weight of the birds. The weight gain was calculated by subtracting the initial weight from the final weight of the bird.

Feed conversion ratio

The feed conversion ratio was calculated by dividing the feed intake per bird per week by average egg weight per week multiplied by number of eggs laid per bird per week.

Feed Conversion Ratio = $\frac{\text{Feed intake per week (g)}}{\text{Average egg weight per week} \times \text{Number of eggs laid per week}}$

Average egg weight per week x Number of eggs laid per week

Egg weight

Eggs were collected from each replicate daily throughout the experimental period to evaluate the egg weight. The egg weight was measured using a sensitive weighing scale.

Hen day production

Hen day production was obtained by dividing the number of eggs collected per

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week by number of days in a week multiply by numbers of birds per treatment.

Hen-day production (%) =

$\frac{\text{Total Number of eggs collected per week}}{\text{Number of days in a week} \times \text{Number of birds}} \times 100$

Egg quality evaluation

At day 42 of the experiment, the egg quality parameters were measured. Egg length and width were measured using vernier caliper. The width was measured as the distance between the broad and narrow ends of the eggs. Egg Shape Index was calculated as the percentage of the egg width to the egg length.

Egg Shape Index = $\frac{\text{Width of egg (mm)}}{\text{Length of egg (mm)}} \times 100$

Yolk height and yolk width were measured using a vernier caliper. Albumen height was measured with tripod micrometer after the egg was gently broken and the maximum height was taken. Yolk index was estimated from ratio of yolk height to yolk width. Yolk colour was determined with a yolk colour fan scale (1 to 15). Haugh unit is a relationship between egg weight and height of thick albumen surrounding yolk. This was calculated using the values obtained from the egg weight and albumen height. This can be expressed as:

$$\text{HU} = 100 \log [H + 7.57 - 1.7W^{0.37}]$$

Where, H = Albumen Height (mm) and W = Weight of the egg (g)

Microbial load

Sample collection and preparation

Two birds were randomly selected and slaughtered from each treatment. The ileum (the portion of the small intestine extending from the *Vitelline diverticulum* to read a point of 40mm proximal to the ileo-caecal junction) was removed. The ileal digesta samples were transferred into syringe, packed in ice and taken to the Environmental Microbiology and Biotechnology Laboratory, Department of Microbiology, University of Ibadan for microbiological analysis.

Laboratory work benches were sterilized using cotton wool dipped in 70% ethanol. All procedures were carried out under aseptic conditions. The culture media used in this study were weighed out following the manufacturers' instructions, and sterilized at 121°C inside an autoclave for 15 minutes. The media used included nutrient agar, eosin Methylene blue (EMB) and MacConkey agar (Oxoid, UK).

Enumeration of the bacterial load of the intestinal content

The enumeration of the bacterial load in the samples was done using the pour plate technique of Harrigan and MacCance (1966). Aliquot (1mL) of the semi-solid intestinal digests was dispensed into 10 mL of sterile peptone water, used as the stock. Ten-fold serial dilution was carried out on the sample using peptone water as the diluent. Aliquots (1mL) of the selected dilutions were plated out on the Nutrient agar (for the Heterotrophic bacterial count) and Eosin Methylene Blue (for the *E. coli* count), MacConkey agar (for the Total coliform). The set up was allowed to set and incubated at 35-37°C for 24-48 hours in the incubator. The plates were observed after the incubation period and observations were noted and recorded.

Chemical analysis

Proximate analysis of the experimental diets was done to determine the moisture content, crude fiber, ash content, crude protein and ether extract according to the methods described by AOAC (2005).

Statistical analysis

Data obtained were analyzed using descriptive statistics and ANOVA, using completely randomized design of Statistical Analysis System (SAS, 2008). The treatment means were separated using Duncan's Multiple Range Test (Duncan, 1955) of the same software package to test significant differences ($p = 0.05$) among the means. Logarithmic ($\log_{10}$) transformation was applied for microbial

colony forming unit (cfu) data.

Results

Performance characteristics of birds fed with humic acid supplemented diets

The results of performance characteristics of laying birds fed humic acid diets are shown in Table 1. There were no significant ($P>0.05$) differences observed in initial weight, feed intake, egg weight, feed

conversion ratio and hen day production. However, the final weight and weight changes were significantly ($P<0.05$) influenced by humic acid supplementation. Birds on 0.4% humic acid supplemented diet had the highest final weight (1721g/bird) and weight gain (97.83g/bird). A decrease in weight was observed in birds fed with 0% humic acid (-168.17g/bird) and 0.2% humic acid (-24.17g/bird) supplemented diets.

Table 1. Performance characteristics of laying hens fed humic acid supplemented diets

Parameter	0% humic acid	0.2% humic acid	0.4% humic acid	SEM	P value
Initial weight (g/bird)	1570.67	1540.33	1623.17	31.93	0.2121
Final weight (g/bird)	1402.50 ^b	1516.17 ^b	1721.00 ^a	49.06	0.0012
Weight change (g/bird)	-168.17 ^c	-24.17 ^b	97.83 ^a	45.58	0.0033
Feed intake (g/bird)	637.59	651.30	639.07	7.49	0.3886
Egg weight (g)	54.83	58.16	57.83	0.52	0.1642
Feed conversion ratio	2.31	2.27	2.27	0.12	0.9570
Hen day production (%)	72.88	70.64	70.64	2.93	0.8238

Means on the same row with different superscripts are significantly ($P<0.05$) different

Internal and external egg attributes of laying hens fed humic acid supplemented diets

The result of humic acid inclusion on the attributes of internal and external qualities of egg is presented in Table 2. There were no significant differences ($P>0.05$) observed in yolk weight, yolk index, albumen height, albumen width, egg length, egg width and egg shape index. However, yolk height, yolk width, yolk colour, Haugh unit and shell weight were significantly ($P<0.05$) influenced across the treatments. Highest ($P<0.05$) yolk height and yolk width were recorded in supplemented diets as compared with the control diet. Lowest yolk colour (2.33) was found in the control diet

while highest (4.16) in 0.4% humic acid diet. Similarly, highest ($P<0.05$) Haugh unit (78.09) and shell weight (7.83g) were recorded in 0.4% humic acid supplemented diet.

Intestinal microbial load in laying hens fed humic acid supplemented diets

The result of humic acid supplementation on the gut microbial load of birds is shown in Table 3. There were no significant ($P>0.05$) differences observed in total coliform count and total *E. coli* count in birds on experimental diets. Least total heterotrophic count (1.10×10^2 cfu/ml) was recorded in 0.2% humic acid diet while highest total Lactobacilli count (0.23×10^2 cfu/ml) was observed in 0.4% humic acid supplemented diet.

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Table 2. Internal and external egg quality parameters of laying hens fed humic acid supplemented diets

Parameter	0% humic acid	0.2% humic acid	0.4% humic acid	SEM	P value
Yolk weight (g)	13.00	13.00	13.33	0.10	0.5486
Yolk height (mm)	3.87 ^b	3.98 ^a	4.00 ^a	0.04	0.0508
Yolk width (mm)	3.69 ^b	3.87 ^a	3.90 ^a	0.04	0.0206
Yolk colour	2.33 ^c	3.16 ^b	4.16 ^a	0.26	0.0163
Yolk index	1.04	1.03	1.02	0.01	0.8145
Albumen height (mm)	5.54	5.54	6.10	0.10	0.1756
Albumen width (mm)	5.46	5.46	5.03	0.11	0.4297
Haugh unit	74.85 ^b	73.56 ^b	78.09 ^a	0.73	0.0212
Egg length (mm)	5.36	5.46	5.23	0.04	0.3398
Egg width (mm)	4.26	4.35	4.34	0.02	0.2737
Egg shape index	0.79	0.79	0.83	0.01	0.2618
Shell weight (g)	6.50 ^c	7.33 ^b	7.83 ^a	0.12	0.0179

Means on the same row with different superscripts are significantly (P<0.05) different

Table 3. Microbial load ($\times 10^2$ CFU/ml) in laying hens fed humic acid supplemented diets

Parameter	0% humic acid	0.2% humic acid	0.4% humic acid	SEM	P value
Total Heterotrophic Count	2.16 ^a	1.10 ^b	1.75 ^a	0.34	0.0521
Total Coliform Count	1.50	1.43	1.44	0.06	0.8998
Total <i>E. coli</i> Count	1.20	1.01	1.15	0.12	0.8678
Total Lactobacilli Count	0.13 ^b	0.17 ^b	0.23 ^a	0.02	0.0378

Means on the same row with different superscripts are significantly (P<0.05) different

Discussion

Humic acid has been reported to improve performance of poultry through healthy gastrointestinal tract ecosystem (Taklimi *et al.*, 2012). However, there have been inconsistencies in reports on the beneficial effects of humic acid in laying hens. These inconsistent results and different responses of supplementary humic acid might be due to the species, age, sex, plane of nutrition, nutrient composition of the diet, levels of humic acid in the diet, stage of production, the duration of supplementation or environmental conditions. In the current study, humic acid had no significant effect on feed intake, egg weight, feed conversion ratio and hen day production except for final weight and weight changes. There was a depression in the weight of hens on the

control diet and 0.2% humic acid supplemented diet, but significant improvement in birds fed with 0.4% humic acid supplemented diet. The positive impact of humic acid on body weight may be due to its effect on stabilizing the intestinal microflora and thus ensures an improved utilization of nutrients in the feed, which could lead to an increase in the live body weight of laying hens (Shermer *et al.*, 1998; Humintech, 2004). Result of the present study is in accordance with the studies on the positive effect of humic acid supplemented diets on body weight gain of broiler chickens and quails (Kocabagli *et al.*, 2002; Abdel-Mageed, 2012). However, Karaoglu *et al.* (2004) and Rath *et al.* (2006), indicated that diets containing humic acid did not significantly affect

broiler chicken's growth performance. In some studies, humate supplementation of the diets of laying quails (Yalçin *et al.*, 2005), broilers (Yalçin *et al.*, 2003) did not improve the body weight at the end of the experiment. There was no difference in feed intake of laying birds placed on humic acid supplemented diet and the control diet. This shows that the birds tolerated humic acid supplementation in their diets. Similar results were reported by Yoruk *et al.* (2004); Hanafy and ElSheikh (2008); Sahin *et al.* (2011); Abdel-Mageed (2012) and Samya (2016). These authors averred that feed consumption was not affected significantly by humic acid supplementation in broiler chickens, laying hens, turkey hens and quails. In addition, Yoruk *et al.* (2004) asserted that humate with concentration of 0.1 and 0.2 % had no significant effect on feed intake in the late stage of laying hens. Kocabagli *et al.* (2002) also indicated that humate diets had no positive influence on feed consumption of broiler chickens. However, some researchers reported that dietary humic acid supplementation increased feed intake in laying hens (Hayirli *et al.*, 2005). On the other hand, Kucukersan *et al.* (2005) showed that the average daily feed consumption of hen fed diets with humic acid was significantly decreased compared with the control group and there was no statistically significant difference in egg weight between the groups.

The results of this current study shows that there was no significant difference in the feed conversion ratio of birds. This agrees with the report of Sahin *et al.* (2011) and is in disagreement with the findings of Yoruk *et al.* (2004), which shows that humates promote growth by improving feed conversion ratio. Mirnawati and Marlida (2013) reported that humic acid at a level of 100 ppm in water improved feed conversion ratio in broilers. Also, Samya (2016) reported best feed conversion ratio during all age intervals of birds fed with humic acid

supplemented diets compared to control diet. Parks *et al.* (1986) reported improved digestibility of nutrients, immunity and growth performance of turkey when fed with humate with low and high crude protein diets.

Regardless of humic acid supplementation, overall mean of egg weight and hen day production were not significantly affected in this study. However, it has been reported that addition of humate into layer diets at a level of 30 and 60 mg/kg (Kucukersan *et al.*, 2005), 90 ppm (Ozturk *et al.*, 2012), up to 0.3 g/kg (Hayirli *et al.*, 2005) or 2 g/kg (Kucukersan *et al.*, 2004) improved egg production and egg weight. On the other hand, Wang *et al.*, (2006) indicated that the dietary humic substances at 5 or 10 % decreased egg production but egg weight was improved.

Yoruk *et al.* (2004) found that supplementation of humate in laying hens diets at 0.1 and 0.2 % for 75 days during the late laying period caused egg production increase compared to control group, while, no significant effect on egg weight was observed. In agreement with the present study, Kucukersan *et al.* (2005) and Yalçin *et al.* (2005) reported that humate supplementation had no effect on the egg weight of laying hens and quails.

The inclusion of humic acid did not influence most of the egg quality attributes measured except for yolk height, yolk width, yolk colour, shell weight and Haugh unit. This is in agreement with the findings of Hanafy *et al.* (2008), who reported no significant differences in egg shape index, albumen percentage and yolk index with humic acid supplementation across the treatments.

The conjugate base of humic acid as used by Macit *et al.* (2009), reported that yolk colour improved with the supplementation of different levels in layer diets, which is similar with the findings of this study but in contrast with the reports of Dobrzański *et al.*

(2009), that yolk colour did not improve with supplementation of humic acid. In the present study, improved Haugh unit was observed in 0.4% humic acid diet, which is contrary to the findings of Abo-Egla *et al.* (2011) who reported no significant effect of humic acid on egg shape index, albumen (%), Haugh unit and egg yolk index when 0.1, 0.2 and 0.3% humic acid was fed to laying hens. According to Arafat *et al.* (2015), humic acid supplementation did not positively influence the Haugh unit and albumen height of laying hens when 2.0, 4.0 or 6.0 mg of humic acid per kg was added in drinking water.

Acidification of diets with weak organic acids has been reported to decrease the colonization of pathogens (Kirchgessner and Roth, 1988). Scientific findings on the impacts of humic substance on gut health and composition of microbiota in the gastrointestinal tract have been reported (Shermer *et al.*, 1998; Tikhonova *et al.*, 2010; Mudroňová *et al.*, 2020). Results of the current study show that feeding 0.4% humic acid supplemented diets had significant effect on the total Lactobacilli. This is similar to the findings of Mudroňová *et al.* (2020) on slight increase in lactic acid bacteria count and decrease in Enterobacteria counts when broiler chicks were fed 0.8% humic substances. The results of this study are at variance with the findings of Pirgozliev *et al.* (2008), on reduction in the population of coliform and Lactobacillus counts in the ileum and caeca of broiler chickens fed with organic acid supplemented diet. Also, Ramarao *et al.* (2004) reported reduction in coliform count in broiler chicken when fed with acidifier at the rate of 300 g/100 kg feed.

Conclusion

This study has demonstrated the effect of dietary humic acid supplementation on laying performance, egg quality parameters and microbial load of egg-type chicken. The

results show that 0.4% humic acid supplementation improved final weight, body weight gain and some egg attributes of laying hens. An increased Lactobacillus count in humic acid diet positively correlated with enhanced gut health of the birds. Therefore, humic acid supplementation up to 0.4% should be encouraged, as this could serve as a viable alternative to antibiotic growth promoter for improved production performance and enhanced gut integrity of laying hens.

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