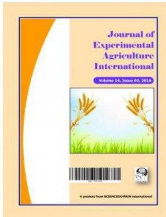

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Growth Performance and Nitrogen Value of Broiler Finisher Birds (29-57 days) in Low-Protein Diets Supplemented with the Most Limiting Essential Amino Acids

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Authors' contributions

This work was carried out in collaboration between all authors. Author SOO led other authors in supervising the designed study. Author FTO was an undergraduate student undergoing project work. Results, statistical analyses and discussion of the research findings were done by all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study evaluated the growth performance and nitrogen utilization of broiler birds of Arbor Acre heavy strain (29-57 days) in which ideal protein concept was tested using the most limiting essential amino acids (EAAs), L-Lysine, DL-Methionine, L-Tryptophan and L-Threonine. Six dietary treatments in which crude protein varied from 20.0% to 8.0% were used to feed broiler birds at the finishing phase (29-57 days) of production. Growth parameters and nitrogen retention were investigated and data obtained were analysed statistically using Minitab (Ver. 16). The results obtained indicated the broilers raised on the 17.0% crude protein diet had the highest significant ($P<0.05$) average feed intake (FI) of 108.13± 0.74 g/bird/day but had feed conversion ratio (FCR) of 2.82± 0.01 similar to diet in which CP was conventionally fixed at 20.0% with minimum EAAs supplementation. The best protein efficiency ratio (PER) of 3.80±0.03 obtained for broilers on the

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least CP level of 8.0%. The average nitrogen retention (NR) values were similar (P>0.05) and higher than other values at 3.06±0.01 g N and 3.01±0.01 g N for birds on the control diet and birds on 20.0% CP with minimum EAAs supplementation, respectively. The average apparent nitrogen digestibility (AND) value of 76.37±0.18% was the highest and significant (P<0.05) for birds on 20.0% CP with EAAs supplementation. Growth parameters for birds on conventional control diet had the best FCR. However, birds reared on 17.0% CP diet with EAAs supplementation had similar growth parameters with birds reared on another 20.0% CP diet with EAAs supplementation. Crude protein can therefore be reduced by 3 percentage points without any detrimental effects on the growth performance indices of broiler finisher birds (29-57 days). However, the poor nitrogen retention (NR) and apparent nitrogen digestibility (AND) for birds on the low protein diets is noteworthy.

Keywords: Low-protein diets; most limiting essential amino acids; nitrogen retention.

1. INTRODUCTION

The application of the concept of the ideal protein in feed formulation makes it possible to adjust the supply of the indispensable amino acids to the requirements of animals in order to avoid deficiencies and limit excesses. These excesses, which are mainly caused by protein-rich raw materials, such as soybean meal, provide some amino acids which go beyond the animal's requirements; they have therefore to be catabolised by animals and they are at the origin of the excretion of nitrogen compounds which are transformed into nitrates in the environment. In the implementation of the concept of the ideal protein, feed-use amino acids are indispensable ingredients to optimize the balance of amino acids in feed formulas – meeting objectives of performance, profitability and respect for the environment. The reduction of nitrogen input to farms, reduces protein diets and lower the nitrogen output to the environment for instance in broiler, a reduction of 2 points of dietary protein content result in a 13% to 14% reduction in nitrogen excretion [1,2].

In addition, health conditions can be improved by reducing the dietary protein supply. The crude protein level is reported as a predisposing factor for necrotic enteritis [3,4].

Moreover, an excess of protein would lead to physiological need for an increase in water consumption to achieve efficient nitrogen excretion. As a consequence, high crude protein diets lead to higher level of nitrogen and water excretion compared with low crude protein diets, leading to deterioration in litter quality and the bird's environment. This study was therefore designed against the background that further searchlights should be beamed at the need to reduce the protein intake and emphasize the ideal protein concept of optimizing the balance of amino acids in broiler production.

2. MATERIALS AND METHODS

2.1 Experimental Site

The experiment was carried out in the Poultry Unit of the Teaching and Research Farm (T & RF) of Ekiti State University, Ado-Ekiti with geographical coordinates of 7° 38'0" North, 5° 13' 0" East The T &RF and a tropical humid climate with distinct wet and dry seasons. The rainy season spans over seven months starting from March/early April to October with a dry spell in August. Temperature in this area is fairly uniform throughout the year with little deviation from the mean annual of 27°c. The topography is moderately sloppy with the highest point having the slope of not greater than 6%. The main vegetation is grass but activities like bush fallowing influences vegetation. The experiment was carried out between March and June, 2016. Further laboratory analyses were carried out at the Animal Production and Health Sciences Laboratories of Ekiti State University and The Federal University of Technology, Akure.

2.2 Site Preparation

Prior to the arrival of broiler chicks, the poultry house and metabolism cage were thoroughly washed and fumigated with diskol (a disinfectant containing 4% benzalkonium chloride, 3% glutaraldehyde, 14% formaldehyde, stabilizers, antioxidants and activators). The house was covered to prevent heat loss and brooding equipment installed.

2.3 Management of Experimental Birds and Experimental Design

A total of 300 day-old broiler chicks of the anak heavy strain were purchased from a reputable hatchery. All chicks were brooded using the manual charcoal heater at the Teaching &

Research Farm of Ekiti State University, Ado-Ekiti, Nigeria. Sex determination was conducted on the chicks on the second day of brooding as described [5]. Clean drinkable water was also provided *ad libitum* with appropriate antibiotics and anti-stress particularly after arrival. Standard medications were administered and veterinary routines were followed. The experiment was conducted as a completely randomized design with a total of two hundred and eight (288) Arbor Acre heavy broiler chicks strain randomly distributed into 6 treatments. Each treatment was replicated four times with each replicate containing 12 birds. The randomization of the broiler chicks was done such that the average weights of all groups were similar at the beginning of the experiment with each replicate containing equal number of male and female chicks. The birds were allowed to feed *ad libitum* throughout the experimental period. Records on daily feed consumption and 3-dayperiodic weight changes were recorded. Four birds were randomly selected (2 males and 2 females) from each treatment such that at least a bird comes from the 4 replicates of each treatment 5 days before the termination of the experiment to determine the nitrogen retention of birds on each diet. These birds were transferred into metabolic cages where the excreta could be collected for analyses.

2.4 Estimation of Nitrogen Retention, Apparent Nitrogen Digestibility and ‘Operative’ Protein Efficiency Ratio

A total of 24 birds were used for the digestibility trial. One bird was taken from each replicate of each treatment making 4 birds from 4 replicates of each treatment. The four birds from each treatment were ensured to be 2 males and 2 females. The 24 birds were placed individually inside separate compartments of the metabolic cage. Total excreta voided during the last 5 days were collected, weighed, dried at 65-70°C in an air circulating oven for 72 h and preserved while the corresponding feed consumed was also recorded for nitrogen studies. The nitrogen contents of the samples were determined [6]. Nitrogen retained was calculated as the algebraic difference between nitrogen intake and faecal nitrogen (on dry matter basis) for the period. Apparent nitrogen digestibility was computed by expressing the nitrogen retained as a fraction of the nitrogen intake multiplied by 100. The protein efficiency ratio was calculated as the ratio of weight gain to total protein consumed.

2.5 Sourcing of Pharmaceutical Feed-Grade Amino Acids

Feed-grade L-Lysine, L-Tryptophan and L-Threonine amino acids were ordered from Ajinomoto Animal Nutrition, Ajinomoto North America, Inc., 4020 Ajinomoto Drive, Raleigh, USA. Pharmaceutical-grade amino acids are reputed to be between 99% and 100% pure. Ajinomoto amino acids are used for multiple nutritional applications, including intravenous solutions, infant and pediatric formulas and other dietary supplements. They are on the list of U.S. Food and Drug Administration (FDA) having complied with the current Good Manufacturing Practice (cGMP). Ajinomoto is a research leader in the nutritional physiology of amino acids, generating knowledge and understanding of the properties and behavior of amino acids, and creating even more medical and nutritional applications. DL-Methionine was purchased locally from a reputable feed mill in Akure, Ondo State, Nigeria.

Table 1. Requirement for crude protein and the most rate limiting amino acids for broilers [7]

Nutrients, %	Weeks of Age		
	0 -3	3-6	6-8
Crude protein	23.00	20.00	18.00
Methionine	0.59	0.38	0.32
Total sulfur			
Amino acids	0.90	0.72	0.60
Lysine	1.10	1.00	0.85
Threonine	0.80	0.74	0.68
Tryptophan	0.20	0.18	0.16
Isoleucine	-.80	0.73	0.62
Arginine	1.25	1.10	1.00
Valine	0.90	0.82	0.70

2.6 Experimental Diets

The feed ingredients used in ration formulation were purchased locally from a reputable commercial feed miller. Feed-grade amino acids were sourced as previously discussed. The experimental diets were compounded and manually mixed on the clean floor of the Poultry Section of the Teaching & Research Farm. The dietary treatments were made up of the control diet 1 which had an approximate value of 20.0% crude protein of both plant and animal (fish meal) origins with a substantial supplementation of DL-Methionine and L-Lysine. Diet 2 also had 20.0% all of plant origins with four most limiting EAAs.

Table 2. Experimental diets for broilers fed varying crude protein levels with EAA supplementation (finisher phase, 29 – 57 days)

Ingredients	Control Diet 1	Crude protein reduction/amino acids supplementation				
		Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
		20.0% CP	17.0% CP	14.0% CP	11.0% CP	8.0% CP
Maize	65.4	61.1	56.8	57.3	57.1	59.1
Soyabean meal	28.0	32.0	25.3	19.8	13.0	6.0
Fish meal (72% CP)	2.0	-	-	-	-	-
Rice husk	-	-	8.0	10.0	15.0	20.0
Palm oil	-	-	3.0	6.0	8.0	8.0
Bone meal	2.5	2.5	2.5	2.5	2.5	2.5
Oyster shell	1.0	1.0	1.0	1.0	1.0	1.0
Salt	0.3	0.3	0.3	0.3	0.3	0.3
*Premix	0.5	0.5	0.5	0.5	0.5	0.5
EAA supplementation						
Lysine	0.15	1.0	1.0	1.0	1.0	1.0
Methionine	0.15	0.7	0.7	0.7	0.7	0.7
Threonine	-	0.7	0.7	0.7	0.7	0.7
Trytophan	-	0.2	0.2	0.2	0.2	0.2
Calculated composition						
Crude Protein, %	19.9	19.9	17.0	14.1	11.0	7.7
**ME (Kcal/Kg)	2893.3	2889.7	2807.16	2890.74	2871.32	2828.23
Crude Fibre, %	3.9	4.1	3.8	3.4	7.4	9.0
Fat, %	3.8	3.6	3.5	3.3	3.1	2.9
Analyzed/chemical composition						
Crude Protein, %	20.1	20.1	16.7	14.3	11.2	7.9
Crude Fibre, %	4.2	4.6	4.3	3.7	3.3	3.1
Fat, %	4.1	3.9	3.7	3.5	3.2	3.1

**Contained vitamins A(10,000,000iu); D(2,000,000iu); E(35,000iu);K(1,900mg); B12 (19mg); Riboflavin(7,000mg); Pyridoxine(3,800mg); Thiamine(2,200mg); D Panthotenic acid(11,000mg); Nicotinic acid(45,000mg); Folic acid(1,400mg); Biotin (113mg); and trace elements as Cu(8,000mg); Mn(64,000mg); Zn(40,000mg); Fe(32,000mg); Se(160mg); I_2(800mg); and other items as Co(400mg); Choline(475,000mg); Methionine(50,000mg); BHT(5,000mg) and Spiramycin(5,000mg) per 2.5kg; CP:Crude Protein, ME:Metabolized Energy.*

***ME, metabolizable energy = (0.860+0.629(GE-0.78CF) [9]*

Diets 3, 4, 5 and 6 contained reduced inclusion levels of crude protein of plant origin at approximate values of 17.0%, 14.0%, 11.0% and 8.0%, respectively. In essence, crude protein was reduced by 3 points across the diets from diet 3 to diet 6. The four most limiting essential amino acids [7] in broilers were supplemented as required in the low crude protein diets. Palm oil is used in diets 3 to diet 6 to shore up the calorie level in order to meet the recommended required meatabolizable energy level in broiler finisher diet.

2.7 Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using Minitab Analytical Computer Package [8]. Duncan's Multiple Range Test was used to separate significant differences among the means and the separated mean values were compared where necessary at a level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Growth Performance

The growth performance of chicks fed varying crude protein levels with essential amino acids (EAAs) supplementation at broiler finishing phase (29-57 days) is presented in Table 3.

The average feed Intake (FI) had the highest significant ($P<0.05$) value of 108.13±0.74 g/b/d for birds on 17.0% CP diet (diet 3) followed by FI value of 98.56±0.39 g/b/d obtained for birds on 14.0% (diet 4). The lowest average value of 70.93±0.18 g/b/d was obtained for birds on 8.0% CP diet (diet 6) and significantly different ($P<0.05$) from all other FI values.

The average weight gain (WG) of 50.26±0.23 g/b/d was significantly higher (P<0.05) than all other WG values. The lowest WG value of 21.68±0.27 g/b/d was obtained for birds on the 8.0% CP diet (diet 6) and this was significantly lower (P<0.05) than all other WG values. The feed conversion ratio (FCR) was lowest and optimum at 1.94 ± 0.01 for birds on the control diet and was significantly different (P<0.05) from all other values. The FCR value of 2.57±0.03 obtained for birds on the 11.0% CP diet (diet 5) was next in decreasing order. The FCR values of 2.81±0.01 and 2.82±0.01 were similar (P>0.05) for birds on 20.0% CP (diet 2) and 17.0% CP (diet 3). The FCR value of 3.27±0.05 was the highest significant (P<0.05) value obtained for birds on 8.0% CP (diet 6).

The protein efficiency ratio (PER) varied significantly (P<0.05) among birds across the six diets. Birds on 8.0% CP diet (diet6) had the highest (P<0.05) PER of 3.80±0.03 followed by 3.53±0.03 obtained for birds on the 11.0% CP diet (diet5). Birds on the control diet 1 had a PER value of 2.60±0.03 while birds on diet 4 (14.0% CP), 5 (14.0% CP) and 2 (20.0% CP), respectively in decreasing order of 2.37±0.03, 2.09±0.03 and 1.80±0.02.

The results obtained from the present study with broiler finisher birds opened a relatively new vista of knowledge into ideal protein concept in broiler production as it was rather curious that average feed intake (FI) even though highest and significant (P<0.05) for birds at 17.0% CP diet still had a feed conversion ratio (FCR) similar to diets in which CP was conventional at 20.0% with essential EAA supplementation. The average FCR for birds on 11.0% CP diets (2.57±0.03) was also curiously lower and better than the FCR values obtained for birds on every other diet except the control diet in which animal protein (fish meal) was used with moderate conventional addition of only methionine and lysine. Depressed feed intake in the low crude protein diets in the present study has been previously reported [10] and appears to partially explain the negative effect of low crude protein diets in some cases. The protein efficiency ratio (PER) surprisingly had the highest value of 3.80±0.03 for birds on the least CP diets of 8.0%.

The relative poor growth performance of broiler finisher birds on the low protein diets have been a subject of controversy in several previous studies [11,12,13,14,15,16,17]. However, it is clear that chickens fed low-protein feeds, despite having enough of each essential amino acid (to support excellent growth) may still fail to thrive because chickens require the essential amino acids plus some other amount of nonessential amino acids to synthesize protein at acceptable rates [18,19,20]. Therefore, it was clear that chickens require not only the essential amino acids but also some other quantity of amino acids, which have been referred to as the "nonessential" amino acids [20]. Clearly, some quantity of these nonessential amino acids is needed (essential) for growth. Practically, the sum of the essential and nonessential amino acids in feedstuffs used for feed formulation in monogastric animals and poultry in particular, is the CP requirement for the particular class of animal and may difficult to have a complete supplementation of these essential and nonessential amino acids when crude protein is significantly reduced in practical feed formulation.

Furthermore, it has long been recognized [21] and further established [7] that the amino acid requirements of the bird are proportional to the CP content of the diet. It has been proved that amino acids are needed in direct proportion to the dietary protein level [11,12,13,14,15,16,22]. Apart from calorie intake and other notable factors, dietary protein level has been highlighted as another determinant of feed consumption [11,12,13,14,15,16,23]. This explains the remarkably poor feed intake values obtained for birds on 11.0% CP and 8.0% CP in the present study.

The present result indicating a similar growth performance indices for birds when 17.0% and 20.0% CP diets supplemented with essential amino acids were fed to experimental broiler chickens at finisher phase corroborated earlier result and conclusion that the CP levels in broiler diets can be reduced by 3 to 4 percentage points without sacrificing performance provided that free amino acids are supplemented in the diet to equal the amino acid levels in a conventional diet [19]. Earlier experiments [19] were all based on the hypothesis that CP levels could be decreased by supplementing synthetic amino acids. Perhaps the most elucidating and corroborative study was one in which the data suggest that after the starter period, reducing dietary CP to 90% of the recommended values had no detrimental impact on broiler performance, with the best result obtained when diets supplemented with Thr at the level of 110% of recommended values [24]. The assumption here is that higher crystalline amino acids content of diet may result in better amino acid availability and better performance because it is assumed that the availability of free crystalline amino acid is higher than that of amino acids in intact proteins.

The high average protein efficiency ratio (PER) values of 3.80±0.03 and 3.53±0.03 obtained for birds on the least 8.0% CP and 11.0% CP diets, respectively agreed with a previous study [23] where protein efficiency ratio (PER) among some other parameters investigated were more efficient ($P \leq 0.01$) in the lower-protein diets. It was also reported that nonessential amino acid (NEAA) supplementation significantly ($P \leq 0.01$) decreased the efficiency of N utilization and that reducing dietary CP from 225 to 153 g kg^{-1} decreased N excretion in a highly significant linear fashion ($P \leq 0.001$; $r = 0.73$) [25]. The corollary of the above finding may be assumed to imply that supplementation with limiting EAAs in broiler chicks may increase N excretion.

A more recent study [20] agreed with the present study by the conclusion of his work that CP level is still a significant contributor to variation in both broiler growth and FE, even when purified amino acids are added to the lower CP feeds.

3.2 Nitrogen Utilization

The nitrogen utilization of birds fed varying crude protein levels with EAAs supplementation at broiler finisher phase (29-57 days) is presented in Table 4.

The average nitrogen intake (NI) were similar (P>0.05) at 3.20±0.14 g N, 3.06±0.02 g N and 2.96±0.02 g N for birds on the control diet 1, diet 2 (20.0% CP with EAAs supplementation) and diet 3 (17.0% CP), respectively. The least NI value of 0.92±0.01 g N was obtained for birds on diet 6 (8.0% CP).

The values of nitrogen in droppings were similar (P>0.05) for birds on the control diet and birds on diet 2 (20.0% CP with minimum EAAs supplementation), 3 (17.0% CP), 4 (14.0% CP) and 5 (11.0% CP) all having the same value of 0.04 g N in their droppings. Only birds on diet 6 (8.0% CP) had a significantly lower (P<0.05) average value of 0.03 g N nitrogen in droppings.

The average nitrogen retention (NR) values were similar (P>0.05) and higher than other values at 3.06±0.01 g N and 3.01±0.01 g N for birds on the control diet 1 and diet 2 (20.0% CP with minimum EAAs supplementation), respectively. The average apparent nitrogen digestibility (AND) value of 76.37±0.18% was the highest and significant (P<0.05) for birds on diet 2 (20.0% CP with minimum EAAs supplementation). This was followed by a significant (P<0.05) AND value of 75.16±0.03% obtained for birds on the control diet 1. Birds on diet 3 (17.0% CP) had a significantly different (P<0.05) value of 70.49 ± 0.04 for AND. The lowest significant (P<0.05) AND value of 40.17±0.09% was obtained for birds on diet 6 (8.0% CP).

The feed intakes were similar (P>0.05) amongst birds on the conventional control diet with minimal methionine and lysine augmentation and also birds on 20.0% and 17.0% CP diets with essential EAA supplementation. However, the least NI value of 0.92±0.01 g N was obtained for birds on least CP intake of 8.0% with essential EAAs supplementation.

The nitrogen in droppings of birds on the least 8.0% CP diet had the lowest significant value (P<0.05) of 0.03 g N but there were similarities in the values of nitrogen in droppings for birds on other diets. The present result agreed with the previous works [1] that chicks fed low-protein diets excreted less N (P < 0.001) than did chicks fed the high-protein diets but disagreed with another study that reported a decrease in N excretion in the low-protein-fed chickens [25].

The average nitrogen retention (NR) values were similar (P>0.05) and higher than other values at 3.06±0.01 g N and 3.01±0.01 g N for birds on the control diet 1 with minimal methionine and lysine supplementation and diet 2 (20.0% CP with minimum EAA supplementation), respectively. The average apparent nitrogen digestibility (AND) value of 76.37±0.18% was the highest and significant (P<0.05) for birds on diet 2 (20.0% CP with minimum EAA supplementation).

The poor nitrogen retention (NR) and apparent nitrogen digestibility (AND) for birds on the low protein diets even with essential EAA supplementation agreed with previous work [1] with the report that chicks fed low-protein diets grew slower, used feed less efficiently, and retained less N and more ether extract than chicks fed the control diets (P < or = 0.05), despite additions of crystalline Gln or Asn and despite increased dietary concentrations of crystalline essential and nonessential amino acids.

Table 3. Performance of chicks fed varying crude protein levels with essential amino acids supplementation at broiler finisher phase (29 – 57 days)

Parameters	Control Diet 20.0% CP (Standard diet)	Crude protein reduction/EAAs supplementation				
		Diet 2 20.0% CP	Diet 3 17.0% CP	Diet 4 14.0% CP	Diet 5 11.0% CP	Diet 6 8.0% CP
Av. Feed Intake, g/b/d	97.19±0.23[c]	94.88±0.17[d]	108.13± 0.74[a]	98.56±0.39[b]	92.33±0.21[e]	70.93±0.18[f]
Av. Weight Gain, g/b/d	50.26±0.23[a]	33.84±0.23[d]	38.36±0.15[b]	32.72±0.52[e]	35.92±0.33[c]	21.68±0.27[f]
Feed Conversion (feed/gain)	1.94±0.01[e]	2.81± 0.01[c]	2.82± 0.01[c]	3.02±0.03[b]	2.57±0.03[d]	3.27±0.05[a]
Protein Efficiency (PER)	2.60±0.03[c]	1.80±0.02[f]	2.09±0.03[e]	2.37±0.03[d]	3.53±0.03[b]	3.80±0.03[a]

a, b, c, Means within a row with different superscript are significantly different (P<0.05)

Table 4. Nitrogen utilization of chicks fed varying crude protein levels with EAA supplementation at broiler finisher phase (29 – 57 days)

Parameters	Control Diet 20.0% CP	Crude protein reduction/EAA supplementation				
		Diet 2 20.0% CP	Diet 3 17.0% CP	Diet 4 14.0% CP	Diet 5 11.0% CP	Diet 6 8.0% CP
Nitrogen intake (gN/bird/day)	3.20±0.14[a]	3.06±0.02[a]	2.96± 0.02[a]	2.26±0.08[b]	1.67±0.07[c]	0.92±0.01[d]
Nitrogen in droppings (gN/bird/day)	0.04±0.00[a]	0.04±0.00[a]	0.04±0.00[a]	0.04±0.00[a]	0.04±0.00[a]	0.03±0.01[b]
Nitrogen Retention (gN/bird/day)	3.06±0.01[a]	3.01±0.01[a]	2.91±0.01[b]	2.18±0.02[c]	1.59±0.01[d]	0.88±0.02[e]
Apparent Nitrogen Dig. (%)	75.16±0.03[b]	76.37±0.18[a]	70.49±0.04[c]	54.30±0.08[d]	45.57±0.06[e]	40.17±0.09[f]

a, b, c, Means within a row with different superscript are significantly different (P<0.05)

4. CONCLUSION

i. The growth parameters for birds on conventional control diet in which 20.0% CP was used with an animal protein source had the best FCR.
ii. However, birds reared on 17.0% CP diet with EAAs supplementation had similar results on growth parameters with birds reared on another 20.0% diet with EAAs supplementation.
iii. Crude protein can therefore be reduced by 3 percentage points from 20% to 17%without any detrimental effects on the growth performance indices of broiler finisher birds (29-57 days).
iv. There were relative poor nitrogen retention (NR) and apparent nitrogen digestibility (AND) for birds on the low protein diets even with essential EAA supplementation.

5. RECOMMENDATION

Broiler production especially at the finisher phase of production can be made more commercially profitable when the cost of synthetic essential amino acids are affordable such that a 3% reduction in the standard recommended crude protein level is achieved in practical feed formulation for broiler birds.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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