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Research Article

Blood Morphology and Antioxidant Status of Broiler Fed *Rhizopus Stolonifer*-Detheobromized Cocoa Pod Husk Meal

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Abstract

This study was carried out to access the blood morphology and antioxidant status of Arbor acre broiler. Four diets were formulated using fermented cocoa pod husk meal at 0, 5, 10 and 15% inclusion levels and labelled as D1, D2, D3 and D4 respectively. Ninety- six day old of Arbor acre broiler consisting of twenty-four birds per treatment of 8 birds per replicate diet group was set up in a Completely Randomized Design (CRD). 5% and 10% fermentation of cocoa pod husk meal (FCPHM) were significantly (P<0.05) higher in PCV (28.33% and 28.67%), MCHC (32.87g/dl and 32.91g/dl), Hb (9.44g/dl and 9.58g/dl) and WBC (5.57 x10^9/l and 5.80 x 10^9/l), respectively. Significantly lower values were observed in D4 (15%) with 25.50%, 31.65g/dl, 8.47g/dl and 3.77 x10^9/l. WBC of 0% was 3.05 x10^9/l is not statistically differed from 15%.The serum indices showed no significant (p>0.05) differences in all parameters except cholesterol and low-density lipoprotein with highest statistical value recorded in 5% FCPHM. Similarly, all the parameters for antioxidant activities of the birds were significantly (p<0.05) affected dietary treatment. From the results, it was concluded conceivably that inclusion of above 10% fermentation of cocoa pod husk mel (FCPHM) in diets of Arbor acre broiler may have a detrimental effect on the health.

Keywords: Antioxidant; Cocoa pod husk; Hematology; *Rhizopus stolonifera*; Serum

Abbreviations: MFCSR: Microbial Fermented Cassava Starch Residue; PCV: Packed Cell Volume; WBC: White Blood Cell; LDL: Low-Density Lipoprotein; MCV: Mean Cell Volume; MCHC: Mean Corpuscular Haemoglobin Concentration

Introduction

The exploitation of agro by-products and farm wastes as substitute feed resources in livestock feeding trials has been the current development in animal production [1] Some of these by-products include wheat and rice offals, maize bran and brewers dried grains; which can switch conventional feed resources in animal diets without harmful effects [2,3]. However, the current prices of these by-products have increased as a result of high demand; thereby demanding the quest for further research into other alternative feed ingredients that are yet to be discovered.

One of such agro by-products that have been exploited in animal feeding trials is cocoa pod husk meal with minute success. Reports have shown that each ton of dry cocoa (beans) epitomizes ten (10) tonnes of cocoa pod husks [4]. Presently, cocoa pod husks are initiating environmental pollution problem in cocoa producing regions of the world. They serve as possible sources of disease transmission when used as mulch in cocoa farms. However, when perfectly processed to reduce the theobromine content and stimulate digestibility; the cocoa pod husk in meal form can be used in livestock feed formulation as a valuable ingredient.

Nutritional value of cocoa pod is relatively low as it is low in crude protein (9.14%) and high crude fibre (35.78%). It also contains anti-nutrients such as theobromine (2.64%), caffeine (1.14%) and tannin 0.917%) [5]. Several methods have been adopted in the treatment and processing of cocoa pod husk meal for the purpose of animal feed formulation. Some of these methods include hot-water treatment [6] alkali treatment [7]; enzyme (mannanase) treatment [8,9]; urea treatment [10,11]; fungal treatment [12], microbial detheobromination [13] and rumen-potash treatment [14].

Omoifo CO [15] reported that *Rhizopus stolonifer*, a Zygomycete has a filamentous growing pattern. Its filaments are coenocytic, that is, they are non-septate. It is the only fungus yet known to yield rhizoids which infiltrate the substratum in order to obtain nutrients. Furthermore, he reported that the rhizoids also serve as support. Opposite the rhizoids, a sporangiophore juts into the atmosphere and this lay off in a club-shaped column lumen closed within the sporangial wall. Between the column lumen and wall are several asexual reproductive structures known as sporangiospores [16]. This organism is also categorized by the presence of stolons, which connect rhizoid joints and it is conceivable that this organism can be used to ferment the cocoa pod husk with a resultant enhancement in the nutritional quality. Thus, cocoa pod husk meal was subjected to solid state fermentation using *Rhizopus stolonifer* fungi in a 14-day fermentation period, characterized chemically and used in formulating diets for broiler chickens to measure the effects of the diets on the blood morphology and antioxidant activity of the use of cocoa pod husk meal fermented with *Rhizopus stolonifer.* The study was geared to ascertain the optimum inclusion level as an ingredient of *Rhizopus stolonifer*- detheobromized cocoa pod husk meal in the diets of broiler chicken on the blood morphology and antioxidant status.

Materials and Methods

Experimental location

The feeding trial was carried out at the Poultry Unit of the Livestock Section, Teaching and Research Farm, The Federal University of Technology, Akure (FUTA). The University (FUTA) is geographically located between latitude 7° 5'N and longitude 5°15'E at an altitude of 370m above sea level [17].

Collection and processing of cocoa (Theobroma cacao) pod husks

Freshly broken composite cocoa pods were obtained from cocoa farm plantations located in Idanre, Ondo State. The broken cocoa pods were washed, milled and fermented *in vitro* using *Rhizopus stolonifer* fungi for 14 days under room temperature. Thereafter, the FCPHM was dried, milled and sample analyzed for proximate composition along with the unfermented sample using the AOAC (2012) procedures.

Cocoa pod husk fermentation method using a starter culture

Fermentation of cocoa pod husk meal (CPHM) was done to reduce the theobromine and fibre contents that could inhibit the utilization of the pod meal in the birds. CPHM was fermented through solid state fermentation during each period of fermentation after milling. Ten (10) grams of urea was dissolved in 100 liters of water which was used to moist the sterilized CPHM. One liter of the prepared inoculums of the starter culture of *Rhizopus stolonifer* was used to inoculate the urea treated CPHM and kept in a tray in an incubating chamber. The fermentation of the cocoa pod husk meal was completed on the 14th day, followed by sun drying the substrates for five to seven days to inactivate the microorganism.

Table 1: Gross composition of the experimental starter diets.

Ingredients	D1 (0%)	D2 (5%)	D3 (10%)	D4 (15%)
Maize	51.3	46.3	42.3	42.3
Wheat offal	4	4	5	5
Soybean meal	21	21	19	16
Groundnut cake	13	13	13	11
Fish meal	5	5	5	5
FCPHM (21%)	0	5	10	15
Lysine	0.1	0.1	0.1	0.1
Methionine	0.1	0.1	0.1	0.1
DCP	2	2	2	2
Limestone	1	1	1	1
Premix	0.25	0.25	0.25	0.25
Salt	0.25	0.25	0.25	0.25
Vegetable oil	2	2	2	2
Total	100	100	100	100
Calculated				
ME (MJ/kg)	12.59	12.61	12.62	12.62
Crude Protein (%)	23.1	23.14	23.19	23.22
Calcium (%)	1.17	1.18	1.18	1.17
Phosphorus (%)	0.73	0.74	0.75	0.74
Lysine (%)	1.28	1.47	1.63	1.72
Methionine (%)	0.48	0.49	0.49	0.48

FCPHM: Fermented Cocoa Pod Husk Meal; DCP: Dicalcium Phosphate; ME: Metabolizable Energy

Table 2: Gross composition of the experimental finisher diets.

Ingredients	D1 (0%)	D2 (5%)	D3 (10%)	D4 (15%)
Maize	59.5	56.5	55	52
Soybean meal	14.5	14	11	9.5
Groundnut cake	16.3	14.8	15.3	14.8
Fish meal	3	3	3	3
FCPHM (21%)	0	5	10	15
Lysine	0.1	0.1	0.1	0.1
Methionine	0.1	0.1	0.1	0.1
DCP	1	1	1	1
Limestone	2	2	2	2
Premix	0.25	0.25	0.25	0.25
Salt	0.25	0.25	0.25	0.25
Vegetable oil	3	3	2	2
Total	100	100	100	100
Calculated				
ME (MJ/kg)	13.36	13.49	13.43	13.56
Crude Protein (%)	20.93	20.88	20.8	20.78
Calcium (%)	1.06	1.06	1.06	1.06
Phosphorus (%)	0.41	0.42	0.42	0.43
Lysine (%)	1.03	1.19	1.32	1.47
Methionine (%)	0.42	0.43	0.43	0.43

FCPHM: Fermented Cocoa Pod Husk Meal; DCP: Dicalcium Phosphate; ME: Metabolizable Energy

Dietary treatment

One basal diet was formulated to meet the nutrient requirement of broiler chicks according to NRC (1994) recommendation. The fermented cocoa pod husks (FCPH) meal was included as an independent ingredient at 0%, 5%, 10% and 15% levels in the diets and were designated as D1, D2, D3 and D4, respectively. The dietary formulae were balanced in such a way that the four diets were isonitrogenous and isocaloric. The formulae for both starter and finisher diets are presented in Tables 1 & 2, respectively.

Chicks arrangement and management

One hundred (100) day-old Arbor Acre broiler chicks were procured from Fidan farms in Ibadan, Oyo State, Nigeria out of which ninety- six (96) were assigned to four (4) dietary treatments of three (3) replicates of eight (8) chicks per replicate. The design of the experiment was a Completely Randomized Design.

Birds slaughtering and blood collection

The birds were fastened 12 hours before the collection of blood early in the morning. Six birds were randomly selected per treatment to determine the carcass and relative organ characteristics, haematological parameters, serum parameters and antioxidants activities. The birds were stunned before slaughtering in compliance with World Poultry Association guidelines. They were bled by severing the jugular vein and carotid artery and blood samples were collected during slaughtering at the end of the experimental period. The blood samples for haematological studies were collected in ethylenediaminetetraacetic acid (EDTA) bottles and blood meant for serum biochemical indices were collected in a test tube without anticoagulants and placed in the slanted form. The blood collected into bottles containing lithium- heparin from each treatment groups were analyzed for oxidative metabolites to ascertain the oxidative stress levels of the experimental birds.

Statistical Analysis

All data that were collected were subjected to One Way Analysis of Variance (ANOVA) of SPSS version 23. Significant treatment means were compared using New Duncan's Multiple Range Test of the same package. The statistical model adopted is as shown below:

$$Y_{ijk} = \mu + T_i + e_{ijk}$$

Where Y_{ijk} = any observation considered, μ= population mean, T_i = effect of FCPHM level,

e_{ijk} = experimental error (assumed to be identical, independently and normally distributed with zero mean and constant variance).

Results

Table 3 shows the haematological parameters of broiler fed varied inclusion levels of fermented cocoa pod husk meal (FCPHM). The dietary treatment recorded significant (p<0.05) differences in PCV, MCHC, Hb and WBC. RBC, MCV and MCH were not significantly (p>0.05) affected with the inclusion levels. D2 and D3 were significantly (p<0.05) higher in PCV (28.33% and 28.67%), MCHC (32.87g/dl and 32.91g/dl), Hb (9.44g/dl and 9.58g/dl), WBC (5.87 x10^9/l and 5.80 x10^9/l), respectively.

Table 3: Haematological parameters of broiler fed varying levels of fermented cocoa pod husk meal.

Parameters	D1	D2	D3	D4	SEM±
Packed Cell Volume (%)	25.83^{b}	28.33^{a}	28.67^{a}	25.50^{b}	0.49
Red Blood Cell (x10^{6}/l)	1.5	1.75	1.88	1.78	0.07
MCHC (g/dl)	32.07^{ab}	32.87^{a}	32.91^{a}	31.65^{b}	0.2
Mean Cell Volume (fl)	187.97	161.93	157.17	152.06	6.45
Mean Cell Haemo (Pg/cell)	62.66	53.98	52.39	50.69	2.15
Haemoglobin (g/dl)	8.64^{bc}	9.44^{ab}	9.58^{a}	8.47^{c}	0.16
White Blood Cell (x10^{9}/l)	3.05^{b}	5.87^{a}	5.80^{a}	3.77^{b}	0.34

a, b & c means on the same row with different superscript is significantly (P<0.05) different. **MCHC:** Mean Cell Haemoglobin Concentration

Significantly lower values were observed in D4 with 25.50%, 31.65g/dl, 8.47g/dl and 3.77 x 10^{9}/l respectively. WBC of D1 (3.05 x10^{9}/l) is not statistically differed from D4.

Serum biochemical parameters as shown in Table 4revealed show no significant (p>0.05) differences in all parameters except cholesterol and LDL. D2 had the highest statistical values of 3.25Mmol/l and 1.49Mmol/l compared to other dietary treatments. HDL, total protein, albumin and AST differed numerically with D1 recording the highest values of 2.95 Mmol/l, 44.07g/l, 12.23g/l and 186.13ui/l respectively. D3 recorded the highest numerical value in globulin with 35.98g/l while D4 also had the highest numerical value of 40.05ui/l in ALT.

Table 4: Serum biochemical parameters of broiler fed varying levels of fermented cocoa pod husk meal.

Parameters	D1	D2	D3	D4	SEM±
Cholesterol (mmol/l)	2.23^{b}	3.25^{a}	2.37^{b}	2.18^{b}	0.15
High-Density Lipo. (Mmol/l)	2.95	2.79	2.86	2.82	0.09
Low-Density Lipo. (Mmol/l)	0.19^{b}	1.49^{a}	0.29^{b}	0.44^{b}	0.15
Total Protein (g/l)	44.07	44.02	46.71	36.26	2.23
Albumin (g/l)	12.23	10.28	10.74	11.01	0.64
Globulin (g/l)	31.84	33.73	35.98	25.25	2.67
AST (ui/l)	186.13	176.45	174.82	174.76	2.89
ALT (ui/l)	38.75	38.43	38.59	40.05	0.45

a, b & c means on the same row with different superscript is significantly (P<0.05) different. **Lipo:** Lipoprotein; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase

Table 5: Antioxidant activities of broiler fed varying levels of fermented cocoa pod husk meal.

Parameters	D1	D2	D3	D4	SEM±
Catalase (u/gHb)	95.18^{a}	79.03^{b}	89.07^{ab}	80.55^{b}	2.1
Superoxide Dismutase (u/ml)	215.47^{ab}	227.03^{a}	207.15^{b}	225.10^{a}	2.53
Glutathione (u/ml)	298.32^{ab}	310.35^{a}	269.02^{b}	300.10^{ab}	6.1

a, b & c means on the same row with different superscript is significantly (P<0.05) different.

Discussion

Haematological parameters have been linked with health indices and are of diagnostic significance in the routine clinical assessment of the state of health [18]. Reports also stated that PCV, HB and MCH were major keys for estimating circulating avian erythrocytes and were very significant in the identification of anaemia and also served as useful parameters of bone marrow capacity to yield red blood cells as in mammals [19].

Akanbi OM [20] reported that haematological components reveal the sensitivity of the animal to its environments which includes feed and feeding. In this study, values of all the haematological indices do not fall within the normal ranges of values except Hb with values of 8.64 – 9.58g/dl compared to normal ranges of values reported by Swenson (1977) of 6.5 – 9.0g/dl. The PCV, RBC and WBC values obtained in this study 25.83 – 28.3%, 1.50 – 1.88 x 10^{6}/l, and 3.05 – 5.87 x 10^{9}/l, respectively were lower than the range of values of 30- 33%, 2.5 – 3.2 x 10^{6}/l, and 9 – 13 x 10^{9}/l, respectively reported by [21]. The FCPHM did not lead to an increase in the WBC but depressed it counts in the experimental broiler chicken. This further reiterates its immunomodulation ability to be low since WBC is concerned in fighting disease-causing organisms and clearing off damaged or dead cells and tissues of the body [22,23]. Similarly, the MCV and MCHC of the birds obtained in this study on the ranges of 152.06 – 187.97 fl and 31.65 – 32.91g/dl, respectively were above the reported range of 127 fl and 29 g/dl, respectively as reported by [24]. The inclusion of FCPHM above 10% recorded lowest values and this indicates that inclusion above this level may have a detrimental effect on the physiological purposes of the birds which could arise from the consequence of theobromine and other

anti-nutrients contained in FCPHM at higher levels. Similarly, the findings of [25] using microbial fermented cassava starch residue (MFCSR) in broilers were closely in agreement with the result of this study.

Neufingerl N, et al. [26] reported that to evaluate the metabolic status of an animal, the serum metabolites levels are vital indicators to verify. In this study, all the serum metabolites measured were not statistically influenced by FCPHM except cholesterol and low-density lipoprotein. Cholesterol, a high molecular weight sterol is used in the body as raw material for a therapeutic process useful in the normal role of the brain and it is an essential constituent of the cell membrane with organelles inside the cell [27]. The present study shows the effect of FCPHM inclusion levels on cholesterol level, though falling within the normal range for healthy chicken did not follow a well- defined trend. Globulin is an important information of blood protein which when present in very low concentration could result in high mortality rate [28]. Sekine S, et al. [29] reported that albumin concentration in serum is established on their factors that are self-determining of nutrition such as infections, liver function, kidney disease, trauma and hydration status which the result of this study clearly shows that none of these extra- nutritional factors had considerable effects on the birds as indicated in the serum antioxidants status of the birds as supported by [25,27] who used MFCSR. 0%, 10% and 15% FCPHM inclusion in arbor acre broiler lessen significantly compared to 5% FCPHM which signifies the possible presence of some bioactive mixtures in FCPHM which impaired fat absorption and consequent fat reduction. The reduced HDL (good cholesterol) and increased LDL (bad Cholesterol) as determined in the result supported the claimed of impaired fat absorption and are also of health aids to the consumers, especially those predisposed to heart diseases. The inclusion of FCPHM in the diet *vis- a- vis* decreased uptake of cholesterol or improved loss or catabolism of cholesterol [30] ALT and AST are significant in the diagnosis of heart liver diseases and also the transamination in the metabolism of precise amino acids. The enzyme (ALT) results in this study were affected by the level of FCPHM inclusion in the diets and did not follow a definite form of effect. This findings is in consistent with [16] when Fungi treated cocoa pod husk meal was tested in the performance and health implication of marshall broilers. The report by [25] findings using MFCSR in broiler birds is in agreement with this present study.

One of the key causes of retarded growth in broiler is oxidative stress under a set of imposed environments. The birds were in very good conditions with physical examination; good toes, well-formed faecal droppings, active behaviour, bright eyes, clean and glossy feathers. Afolabi AB & Oloyede OI [32] reported that the use of herbal established antioxidants to improve the stress is becoming popular and antioxidants significantly delay or prevent oxidation of carbohydrates, Deoxyribonucleic acid (DNA), proteins and lipids. For instance, an antioxidants enzyme like glutathione (GSH), superoxide dismutase (SOD) and catalase can prevent oxidation both by calming transition metal radicals such as Fe^{2+} or Cu^{+} or by foraging instigated free radicals such as superoxide and hydrogen peroxide, the best reactive free radical *in- vivo* [32]. In this study, the inclusion FCPHM did not trail a particular movement in the serum antioxidants. This implies that higher serum antioxidants concentration values recorded for the birds fed diet containing FCPHM had more free radicals- mediated cell damage. The statistically higher results in 5 - 15% FCPHM could be due to the high inclusion level of theobromine content contained in FCPHM. It is, however, noteworthy that the birds fed a diet containing 0% FCPHM had no deleterious effect and less subjected to free radicals- mediated cell damage and oxidative stress. The results obtained in this study are supported by findings of [33-44].

Conclusion

Within the limit of this study, the inclusion of FCPHM above 10% may have a detrimental effect on the haematological, serum biochemical indices and serum antioxidant activities of the broiler upon subjection to stress. Additional research is needed to find further techniques of processing cocoa pod husk so that its theobromine content could be further reduced.

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Availability of Data and Materials

It is available from the corresponding author on reasonable request.

Author's Contributions

JOA designed the study. All authors managed the activities of the experiment and interpreted the data collectively. AOM and MO prepared the proposal of the study. AOM and AOA prepared the first draft of the manuscript. JOA reviewed the first draft. AOA prepared the second draft. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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