

# DIET SUPPLEMENTATION WITH YEAST BETA-GLUCANS, DIETARY ANTIOXIDANTS AND VITAMIN K IN BROILER CHICKENS MITIGATED AFLATOXIN-INDUCED GROWTH RETARDATION

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## ABSTRACT

Mitigation of inevitably absorbed aflatoxins following the addition of toxin binders are insufficiently reported. Therefore, effect of yeast beta-glucans, antioxidants such as vitamins C (VC) and E (VE), selenium (Se) and anti-haemorrhagic agent (vitamin K- VK) in the diet on changes in biochemical processes that characterised aflatoxins poisoning in broiler chickens was investigated. *Aspergillus flavus* 3228 inoculated maize was used to formulate a basal diet (BD) containing  $270 \pm 16 \mu\text{g} \cdot \text{kg}^{-1}$  total aflatoxins (AFB1 and AFB2). Unsexed one-day-old Arbor-Acres broiler chicks ( $n = 180$ ) were randomly assigned into six dietary treatments, comprising Negative Control (NC- aflatoxin-free diet), BD and BD containing beta-glucans, VC, VE, VK and selenium. Two levels of beta-glucans: 250 and 375  $\text{mg} \cdot \text{kg}^{-1}$  (BD250 and BD375) and two combinations of vitamins with or without selenium: [(VE + VC) +VK] = ECK and [(VE + VC) + VK + Se] = ECKSe were combined to give BD250 + ECK; BD250 + ECKSe; BD375 + ECK and BD375 + ECKSe. Selenium, VC, VE and VK, were included in the diets at 0.3, 250, 200 and 3.0  $\text{mg} \cdot \text{kg}^{-1}$  of feed, respectively in augmented (2 x 2) +2 factorial arrangement in completely randomised design. The diets were fed to the chicken *ad libitum* for seven weeks. Serum malondialdehyde, reduced (GSH) and oxidised (GSSG) glutathione levels and ratio, body weight gain (BWG) were measured and feed conversion ratio (FCR) and percentage mortality were determined. Data were analysed using ANOVA at  $\alpha 0.05$ . Serum malondialdehyde and GSSG levels were significantly reduced ( $P < 0.05$ ) from  $159.41 \pm 23.68 \text{ nM} \cdot \text{mL}^{-1}$  and  $5.96 \pm 5.20 \mu\text{M} \cdot \text{mL}^{-1}$  in BD, to  $69.68 \pm 26.97 \text{ nM} \cdot \text{mL}^{-1}$  and  $2.21 \pm 0.88 \mu\text{M} \cdot \text{mL}^{-1}$  in birds on BD375 + ECKSe. Birds fed BD375 + ECKSe had GSH:GSSG ( $3.58 \pm 1.71$ ), BWG ( $1,903.98 \pm 32.56 \text{ g} \cdot \text{bird}^{-1}$ ) significantly higher and FCR ( $1.88 \pm 0.04$ ) was reduced ( $P < 0.05$ ) compared to  $1.06 \pm 0.81$ ;  $956.27 \pm 19.34 \text{ g} \cdot \text{bird}^{-1}$  and  $2.38 \pm 0.04$ , respectively, in birds on BD. Birds fed BD375 + ECKSe had no variation ( $P > 0.05$ ) in performance when compared to NC. Mortality decreased significantly ( $P < 0.05$ ) from  $39.39 \pm 5.25 \%$  in BD to  $9.09 \pm 5.25 \%$  in BD375 + ECK. Combinations of 375  $\text{mg} \cdot \text{kg}^{-1}$  beta-glucans, vitamins E, C, K and selenium reduced lipid peroxidation activity, feed conversion ratio and oxidative stress, with increased bodyweight gain. Finally, combinations of beta-glucans, antioxidants and vitamin K demonstrated effectiveness in preventing changes in biochemical processes leading to aflatoxins deleterious effects in broiler chickens.

**Key words:** absorbed aflatoxins; secondary metabolite; performance; lipid peroxidation; oxidative stress

## INTRODUCTION

Increase in poultry productivity and sustainability require among others, feeding of qualitative feed devoid of mycotoxins and other contaminants. Aflatoxins are a group of mycotoxins and are also

structurally related compounds, with low molecular weight. Aflatoxins are secondary metabolites of filamentous fungi, mainly from *Aspergillus* species (Zain, 2010). They are pharmacologically potent toxins to animals and humans at very low concentration (Tian and Chun, 2017). "Aflatoxicosis"

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is a general term used for diseases caused by aflatoxins ingestion and it is basically a hepato-degenerative condition (Peles *et al.*, 2019). Aflatoxicosis results in liver damages, increased mortality (Manafi, 2018) by weakening the immune system (Monson *et al.*, 2015) and production of hepatic oxidative stress effect in animals (Omar, 2013).

Fungi are ubiquitous in nature and are usually associated with crops such as maize, groundnut, cassava, yam, spices and other basic staples of the African diets (N'dede *et al.*, 2012) and animal feeding stuff. Fungi that are associated with aflatoxins production thrives successfully in warm and humid climates – the prevailing climatic conditions in sub-Saharan region of Africa and given also that field control strategies to reduce aflatoxins contamination is at its lowest ebb or almost non-existent. The probability, therefore, that aflatoxins contamination would be unavoidable in maize grains used in poultry feed production is very high. Maize accounts for between 50–65 % of the energy sources in broilers feed. Therefore, there is high probability of having aflatoxins in feed at unacceptable level if the maize is contaminated, and will be a severe risk to poultry health and productivity (Nazhand *et al.*, 2020). Socio-political unrest or situations resulting in vehicular movements restriction creates grains scarcity scenario, leading to the appearance of rejected maize grains or grains of unacceptable quality in the market.

Mycotoxins' binders have been applied to greatly mitigate the induction of aflatoxicosis in poultry. However, toxin adsorption by mycotoxin binders may never be 100 % effective, because the binding capability of toxin binders might be easy to quantify in an *in vitro* assessment. However, the environment surrounding a toxin binder in an *in vivo* study is quite unique and different from test apparatus environment (Kolossova and Stroka, 2011.). Toxin adsorption in *in vivo* studies is influenced by: (a) limitation in the duration the digesta stays within each segment of the gastrointestinal tract (GIT); (b) fluctuation in gut pH; (c) the state of structural integrity of intestinal linings; (d) increase in concentration of a mycotoxin than anticipated and (e) the influence of gut microbes (Čolović *et al.*, 2019).

Also, since mycotoxins are generally low molecular weight substances, their absorption in the GIT occurs by passive diffusion across concentration

gradients (Gratz *et al.*, 2006). This implied that absorption is taking place as feed is being ingested and digested in the GIT, however, mycotoxins binding by probiotics or microorganisms (Vinderola and Ritieni, 2015), and adsorption by organic or inorganic adsorbent (Goncalves *et al.*, 2017) require some time for the binding process to take place, as it is seen during *in vitro* mycotoxins binders' evaluation by Kolawole *et al.* (2019). The simultaneous occurrence of adsorption by binders and absorption into the circulatory system from the GIT, will eventually affect overall toxin binding efficiency *in vivo*. A major shortcoming of mycotoxins binders is their inability to mitigate the inevitably absorbed fraction of the ingested toxin, which may be sufficient to induce deleterious effects, depending on the initial concentration of the toxin in feed. Therefore, this study investigated effects of supplementation with yeast beta-glucans, dietary antioxidants (such as selenium, vitamins E and C) and vitamin K (an anti-haemorrhagic agent) on changes in biochemical processes that characterised the adverse consequences of dietary aflatoxins on the performance of broiler chickens.

## MATERIAL AND METHODS

### Experimental Location

Feeding trial was carried out at the Teaching and Research Farm of the University of Ibadan, Ibadan, Nigeria in the month of August and September. The climate was a Tropical Savanna Climate, with coordinates of 7° 23' N and 3° 55' E. The environmental temperature ranged from 22 °C (minimum) to 36.5 °C (maximum). Humidity was between 87–88 % and daily sunshine hour ranged between 3.5 and 4 hours.

This experiment was part of a Ph.D. thesis, reviewed and approved by the Department of Animal Science, which conformed with ARRIVE guideline (Percie du Sert *et al.*, 2020).

### Experimental Materials

Whole and clean maize grains were inoculated with toxigenic strain of *Aspergillus flavus*, isolate 3228, obtained from the Mycotoxin Laboratory of the International Institute of Tropical Agriculture, IITA, Ibadan, Nigeria. The fungi isolate multiplication

on 5/2 medium (50 ml of V8 juice and 20 g of Bacto agar) to produce sufficient inoculum and the grain culturing process to generate aflatoxins were done in the Department of Animal Science, University of Ibadan, adapting the method of Atehnkeng *et al.* (2008). The contaminated grains were used to produce a basal diet having  $270 \pm 16 \mu\text{g}\cdot\text{kg}^{-1}$  total aflatoxins. Selenium, vitamins C, E and K were provided by Nutrivitas Nig. Limited, Lagos, Nigeria.

### Experimental Animals, Management and Diets

One-day-old (mixed sexes) Arbor Acres broiler chicks ( $n = 180$ ) were divided randomly into six dietary treatments, using GraphPad QuickCalcs – Random number calculators ([www.graphpad.com](http://www.graphpad.com)). Each treatment comprised 30 chicks, replicated three times with 10 chicks per replication in a spacing of  $0.3 \text{ m}^2$  per bird ( $2 \text{ m} \times 1.5 \text{ m}$ ). The chicks were housed in an open sided deep-litter house. Feed and water were provided *ad libitum*, while daily feed intake was determined. Routine vaccination programme was followed and chicks were covered against Chronic Respiratory Disease. Treatment 1 was the negative control-NC or uncontaminated diet (aflatoxin-free diet) while Treatment 2 was the positive control (basal diet- BD, with aflatoxins but beta-glucans, antioxidants and vitamin K-free). Treatments 3–6 were contaminated diets having beta-glucans, antioxidants and vitamin K added to them. However, Treatments 3 and 5 did not contain selenium. Aflatoxin-free diet was produced by using maize grains harvested from aflasafe® treated maize plants while on the field.

### Dietary Treatment Layout

Treatment 1 = Negative control

Treatment 2 = Basal Diet- BD (Positive control)

Treatment 3 = Basal Diet + 250 mg beta-glucans + [(200 mg VE + 250 mg VC) + 3 mg VK] = (BD250 + ECK)

Treatment 4 = Basal Diet + 250 mg beta-glucans + [(200 mg VE + 250 mg VC) + 3 mg VK + 0.3 mg Se] = (BD250 + ECKSe)

Treatment 5 = Basal Diet + 375 mg beta-glucans + [(200 mg VE + 250 mg VC) + 3 mg VK] = (BD375 + ECK)

Treatment 6 = Basal Diet + 375 mg beta-glucans + [(200 mg VE + 250 mg VC) + 3 mg VK + 0.3 mg Se] = (BD375 + ECKSe)

NB: VE- vitamin E, VC- vitamin C, VK- vitamin K, Se- Selenium.

### Feed Aflatoxins Determination and Proximate Analysis

Aflatoxins in feed samples were extracted using AOAC method 968.22 (AOAC,1990) and High-performance thin layer chromatography (HPTLC) with a scanning densitometer (Ramesh *et al.*, 2013), using CAMAG TLC, Scanner 3, with win-CATS 1.4.2 software (Camag AG, Muttenz, Switzerland) for quantitation at the Mycotoxins Laboratory of IITA, Ibadan. The proximate composition of the test diets was done using the methods of AOAC (2000). Feed nitrogen (N) levels were analysed by the Kjeldahl method and crude protein estimated by  $(N \times 6.25)$  (AOAC, 2000).

### Performance Parameters Assessed

Weighed quantity of feeds were offered daily and remnants weighed before another day's feeding. The difference was used to obtain the actual feed intake. Feed conversion ratio (FCR) was calculated as follows:

$$\text{FCR} = \frac{\text{Total feed consumed/bird (g)}}{\text{Body weight gain/bird (g)}}$$

Mortality rate: The percentage mortality was expressed as the percentage of dead birds over the total number of birds in each treatment, replicate wise.

Body weight (BW) Uniformity was calculated following standard procedure by estimating 10 % deviation from the mean. The number of birds that falls in-between this range was expressed as a percentage of birds in each replicate. Uniformity coefficient of variation (CVu) was determined by expressing the uniformity standard deviation as a percentage of the mean. Coefficient of variation in planned experiment gives an idea of the repeatability of the parameter determined.

### Serum Collection and Analysis of Biochemical Indices

At the end of week 7, three birds (one above, one below and one about the mean weight) were selected from each replicate for specimen collection. Blood sample collection was done via jugular venipuncture and 3ml were collected from each bird into a sterile bottle for selected serum biochemical indices analysis. Serum samples were separated with a centrifuge at 4,000 rpm for about 15 minutes. The separated serum was removed

with the aid of needle and syringe, into another sterile bottle and kept frozen at about -20 °C until it was analysed. Aspartate aminotransferase (AST) in the serum samples was determined by the method of Yagi *et al.* (1979), while alanine aminotransferase (ALT) was determined by the method of Hamada and Ohkura (1976). Alkaline phosphatase (ALP) was assayed by the method of Rosalki *et al.* (1993). Plasma malondialdehyde (MDA) was assayed using Elabscience® Malondialdehyde Colorimetric Assay kit (through the TBA method), to estimate free radical/ or lipid peroxidation activity, which can be determined indirectly by reacting breakdown products from lipid peroxidation with thiobarbituric acid (TBA). The manufacturer procedure followed the method of Ohkawa *et al.* (1979), which was used to estimate level of MDA in the serum samples, by measuring the absorbance of the red colour compound that developed at 532nm with spectrophotometer. Total Glutathione (T-GSH) and oxidised glutathione (GSSG) were assayed using the method of Rahman *et al.* (2006). Since Glutathione reductase (GSR) recycled GSSG generated when glutathione peroxidase uses reduced glutathione (GSH) as reductant in neutralising hydrogen peroxide and lipid hydroperoxide, back into GSH, the overall amount of glutathione in the serum was determined by adding both reduced and oxidized glutathione together. That is: ([GSH] total = [GSH] + 2 × [GSSG]) (Rahman *et al.*, 2006).

#### Cost benefit analysis and variables determinations

Variables of cost analysis determined expressed in Nigerian naira (₦) are listed as follows:

**Average Final Bodyweight** – *AFBW*. This is average bodyweight of each treatment at the 49<sup>th</sup> day of the experiment.

**Average Feed Cost** – *AFC*. The AFC per bird was determined by multiplying the quantity of feed consumed in each treatment by the feed cost per kg of that treatment. The costs of beta-glucans, selenium, vitamins C, E and K were also added to the AFC, based on their inclusion rate in each treatment. Dividing this by the number of birds left in the treatment gave rise to AFC per bird.

**Average Total Raising Cost** – *ATRC*. This is the addition of AFC plus other costs incurred in raising the birds. These other costs include: i) cost of day-

old chicks; ii) cost of vaccines and medications; iii) brooding facilities and expenses; iv) miscellaneous expenses such as transportation, disinfectants and others.

**Average Liveweight Value** – *ALWV*. This was based on the average market liveweight value per kg of bodyweight. The ALWV per bird is a product of *AFBW* and liveweight value per kg body weight, expressed in naira (₦).

**Average Marginal Returns** – *AMR*. This is the difference between the *ALWV* and *ATRC*, expressed in naira (₦).

Therefore,  $AMR \text{ per bird} = ALWV \text{ per bird} - ATRC \text{ per bird}$

\*Note: 1₦ is equivalent to 0.0024 USD

#### Histopathology processing and procedures for tissue sections

Nine birds selected from each treatment were stunned together in a closed chamber, by asphyxiating them with about 70 % CO<sub>2</sub>. When observed to be unconscious, each was shackled by the legs and hoisted head down, and immediately bled by sticking knife through the neck to put them to death. The birds were cut open and the liver harvested. At the point of collection, samples were preserved in 10 % formalin solution (neutral buffered). Samples were appropriately labelled and the histological examinations carried out at the Department of Veterinary Pathology, University of Ibadan. In the laboratory, samples were further processed in an automated tissue processor and embedded into paraffin wax. With the aid of a rotary microtome mounted on glass slides, sectioning at 4–5 microns was done. Staining was done with Haematoxylin and Eosin (H & E), routinely used in histopathology to assess changes in animal tissues and organs in toxicity examinations. Slide observation started with the naked eye, then by microscopy for further examination using Olympus CX21 microscope with attached digital camera. Detailed procedure for the automated tissue processor for histopathology of slides as revised by Slaoul and Fiette (2011) was followed.

#### Experimental Design and Statistical Analysis

Experimental layout was a 2 + 2 × 2 augmented factorial arrangement, in a completely randomised design. Analysis of variance (ANOVA) was used to analyse data generated, using SAS (2012) software

package, version 9.20 and descriptive statistics. Differences between significant treatment means were revealed using Duncan's Multiple Range Test (DMRT). Treatment means declared as being significant were based on 0.05 % level of probability.

## RESULTS

### Effect of varied inclusion levels of beta-glucans and selenium on serum biochemical parameters at day 49

Table 1 shows the combined effect of yeast beta-glucans, supplemental antioxidants and vitamin K on some selected biochemical indices of broiler chickens fed aflatoxin-contaminated feed. No significant difference ( $P > 0.05$ ) was observed in AST values across all the treatments. Beta-glucans, supplemental antioxidants with or without selenium and vitamin K, provided a midway improvement in the ALT values between birds fed NC diet and BD. However, the ALT of birds fed all four mitigated diets (BD250 + ECK to BD375 + ECKSe) did not show significant variations ( $P > 0.05$ ) from one another. Significant reduction ( $P < 0.05$ ) was recorded in ALP values in all four mitigated treatments (BD250 + ECK to BD375 + ECKSe) containing beta-glucans, supplemental antioxidants and vitamin K, to a level of no significant difference ( $P > 0.05$ ) when compared to the ALP of birds on NC diet. The MDA, an index of lipid peroxidation activity, was the least in birds fed NC diet. Addition of beta-glucans, supplemental antioxidants and vitamin K significantly reduced ( $P < 0.05$ ) plasma MDA level in birds fed BD375 + ECKSe compared to those of birds on BD. Birds fed BD375 + ECK and BD375 + ECKSe due to higher beta-glucans inclusion had notable reduction in their MDA values (88.78 and 69.68 nM.mL<sup>-1</sup>) compared to birds fed 250 mg.kg<sup>-1</sup> beta-glucans diets and BD. Reduced glutathione (GSH) values were significantly increased ( $P < 0.05$ ) in all treatments containing beta-glucans, supplemental antioxidants and vitamin K. Oxidised glutathione (GSSG) was significantly reduced ( $P < 0.05$ ) in all the mitigated treatment diets compared to those of birds on BD. The reduction in GSSG of birds on mitigated diets were to levels of insignificant differences ( $P < 0.05$ ) when compared to birds fed NC diet. Oxidative stress index (GSH:GSSG), was significantly increased ( $P < 0.05$ ) in birds fed diets having 375 ppm beta-

**Table 1: Effect of varied levels of beta-glucans and selenium supplementation on serum biochemical indices of broilers offered aflatoxin-contaminated poultry feed from 0 to 49 days**

Treatments	Aflatoxins (µg.kg <sup>-1</sup> )	Beta-glucans (mg.kg <sup>-1</sup> )	Se (mg.kg <sup>-1</sup> )	AST (U.L <sup>-1</sup> )	ALT (U.L <sup>-1</sup> )	ALP (U.L <sup>-1</sup> )	MDA (nM.mL <sup>-1</sup> )	T-GSH (µM.mL <sup>-1</sup> )	GSH (µM.mL <sup>-1</sup> )	GSSG (µM.mL <sup>-1</sup> )	GSH:GSSG
NC	0	0	0.00	104.64 ± 13.28	5.00 ± 3.04 <sup>b</sup>	161.21 ± 42.92 <sup>b</sup>	46.77 ± 14.53 <sup>d</sup>	10.46 ± 4.56	7.19 ± 4.47 <sup>a</sup>	2.03 ± 0.83 <sup>b</sup>	3.66 ± 1.84 <sup>a</sup>
BD(PC)	270	0	0.00	107.78 ± 24.63	11.00 ± 6.27 <sup>a</sup>	249.60 ± 6.27 <sup>a</sup>	159.41 ± 23.68 <sup>a</sup>	9.64 ± 7.48	3.50 ± 1.25 <sup>c</sup>	5.96 ± 5.20 <sup>a</sup>	1.06 ± 0.81 <sup>c</sup>
BD250 + ECK	270	250	0.00	118.58 ± 9.41	7.33 ± 3.42 <sup>ab</sup>	196.66 ± 32.76 <sup>ab</sup>	104.05 ± 28.47 <sup>b</sup>	9.70 ± 3.76	5.06 ± 0.82 <sup>b</sup>	2.61 ± 0.71 <sup>b</sup>	2.01 ± 0.33 <sup>bc</sup>
BD250 + ECKSe	270	250	0.30	111.32 ± 15.44	7.68 ± 1.78 <sup>ab</sup>	156.16 ± 66.20 <sup>b</sup>	94.30 ± 13.98 <sup>b</sup>	9.97 ± 5.59	5.68 ± 1.53 <sup>ab</sup>	2.68 ± 0.61 <sup>b</sup>	2.21 ± 0.68 <sup>bc</sup>
BD375 + ECK	270	375	0.00	112.74 ± 16.85	6.88 ± 3.17 <sup>ab</sup>	172.35 ± 61.74 <sup>b</sup>	88.78 ± 17.40 <sup>bc</sup>	7.69 ± 3.53	4.63 ± 0.87 <sup>bc</sup>	1.96 ± 0.20 <sup>b</sup>	2.38 ± 0.49 <sup>b</sup>
BD375 + ECKSe	270	375	0.30	110.86 ± 21.42	8.66 ± 3.32 <sup>ab</sup>	159.28 ± 21.78 <sup>b</sup>	69.68 ± 26.97 <sup>c</sup>	9.85 ± 2.94	6.88 ± 1.43 <sup>ab</sup>	2.21 ± 0.88 <sup>b</sup>	3.58 ± 1.71 <sup>a</sup>
± SEM				6.21	1.33	20.79	7.65	2.06	0.76	0.78	0.0003
P-value				0.72	0.06	0.02	<0.0001	0.95	0.01	0.01	0.40

<sup>abcd</sup>Means of treatments along a column with different superscript differed significantly ( $P < 0.05$ ). Se – selenium, NC – Negative control, PC – Positive control, ± SEM – Standard error of means, P-value – probability level, AST – Aspartate aminotransferase, ALT – Alanine aminotransferase, ALP – Alkaline phosphatase, MDA – Malondialdehyde, T-GSH – Total glutathione, GSH – Reduced glutathione, GSSG – Oxidised glutathione, GSH:GSSG – Oxidative stress/stability index, Se – Selenium, NC – Negative Control, BD – Basal Diet, BD250 – 250 mg.kg<sup>-1</sup> Beta-glucans, BD375 – 375 mg.kg<sup>-1</sup> Beta-glucans, ECKSe – vitamin E; vitamin K and Selenium.

glucans inclusion compared to those of birds fed BD. Birds on BD375 + ECKSe had GSH:GSSG value similar ( $P > 0.05$ ) to those of birds on NC diet.

### Comparative assessment of effects beta-glucans and selenium on the performance of broiler chickens (0–49 days)

Table 2 shows the performance indices of broiler chickens fed aflatoxin-contaminated feed supplemented with beta-glucans, supplemental antioxidants (with or without selenium) and vitamin K. Birds fed BD had significant ( $P < 0.05$ ) reduction in FI while birds on 375ppm beta-glucans diets had similar FI but significantly reduced ( $P < 0.05$ ) when compared to those of birds on NC diet. The BWG of birds on BD was significantly ( $P < 0.05$ ) reduced while birds fed on all diets containing beta-glucans, supplemental antioxidants and vitamin K had progressive improvement in BWG. Birds fed on BD375 + ECKSe had BWG similar to those of birds on NC diet. Significantly higher and undesirable FCR ( $P < 0.05$ ) was observed in birds fed BD while the addition of beta-glucans, antioxidants and vitamin K significantly reduced FCR of birds on BD375 + ECKSe. Birds fed BD had higher ( $P < 0.05$ ) mortality (39.4 %) while all dietary treatments with beta-glucans, supplemental antioxidants and vitamin K had significantly reduced ( $P < 0.05$ ) mortality compared to birds on BD. Birds on BD375 + ECK had similar mortality with birds on NC diet while birds on BD375 + ECK and BD375 + ECKSe showed no significant differences ( $P > 0.05$ ) in their mortality. Body weight (BW) uniformity was higher ( $P < 0.05$ ) in birds on NC diet (96.7 %) but similar ( $P > 0.05$ ) to those of birds on BD375 + ECKSe diet (89.7 %). Birds fed BD had significantly lower ( $P < 0.05$ ) BW uniformity (24.7 %). Uniformity coefficient of variation (CVu) which is an index of to what extent a measured parameter will not be repeatable was significantly higher ( $P < 0.05$ ) in birds on BD (33.77 %) and desirable CVu was obtained in birds on BD250 + ECKSe (9.91 %); BD375 + ECK (12.56 %) and BD375 + ECKSe (6.16 %) diets, which were to a level of insignificant difference ( $P > 0.05$ ) to that of birds on NC diet (4.63 %).

### Comparative Cost benefit analysis

The effect of varied levels of beta-glucans inclusion and supplemental selenium on cost benefit in broiler chicken fed aflatoxin-contaminated feed

**Table 2: Effect of varied levels of beta-glucans and selenium supplementation on performance indices of broiler chickens offered aflatoxin-contaminated poultry feed from 0 to 49 days**

Treatments	Aflatoxins ( $\mu\text{g}\cdot\text{kg}^{-1}$ )	Beta-glucans ( $\text{mg}\cdot\text{kg}^{-1}$ )	Se ( $\text{mg}\cdot\text{kg}^{-1}$ )	FI ( $\text{g}\cdot\text{bird}^{-1}$ )	BWG ( $\text{g}\cdot\text{bird}^{-1}$ )	FCR	Mortality (%)	BW Uniformity (%)	CVu (%)
NC	0	0	0.00	3924.83 ± 91.67 <sup>a</sup>	1966.24 ± 29.77 <sup>a</sup>	1.99 ± 0.04 <sup>bc</sup>	3.03 ± 5.25 <sup>d</sup>	96.67 ± 5.77 <sup>a</sup>	4.63 ± 1.29 <sup>c</sup>
BD (PC)	270	0	0.00	2279.65 ± 81.44 <sup>e</sup>	956.27 ± 19.34 <sup>e</sup>	2.38 ± 0.04 <sup>a</sup>	39.39 ± 5.25 <sup>a</sup>	24.67 ± 15.95 <sup>d</sup>	33.77 ± 8.84 <sup>a</sup>
BD250 + ECK	270	250	0.00	2579.40 ± 33.63 <sup>d</sup>	1230.40 ± 63.58 <sup>d</sup>	2.09 ± 0.10 <sup>b</sup>	21.21 ± 5.25 <sup>b</sup>	38.00 ± 25.16 <sup>cd</sup>	21.08 ± 9.40 <sup>b</sup>
BD250 + ECKSe	270	250	0.30	2801.27 ± 51.03 <sup>c</sup>	1380.55 ± 27.12 <sup>c</sup>	2.03 ± 0.06 <sup>bc</sup>	24.24 ± 5.25 <sup>b</sup>	64.67 ± 9.61 <sup>b</sup>	9.91 ± 0.88 <sup>c</sup>
BD375 + ECK	270	375	0.00	3479.57 ± 108.09 <sup>b</sup>	1647.92 ± 109.68 <sup>b</sup>	2.12 ± 0.15 <sup>b</sup>	9.09 ± 5.25 <sup>cd</sup>	60.00 ± 10.00 <sup>bc</sup>	12.56 ± 5.49 <sup>bc</sup>
BD375 + ECKSe	270	375	0.30	3588.13 ± 38.16 <sup>b</sup>	1903.98 ± 32.56 <sup>a</sup>	1.88 ± 0.04 <sup>c</sup>	12.12 ± 5.25 <sup>c</sup>	89.67 ± 0.58 <sup>a</sup>	6.16 ± 1.87 <sup>c</sup>
± SEM				42.11	32.59	0.05	2.77	7.86	3.36
P-value				<0.0001	<0.0001	0.0002	<0.0001	0.0002	0.0004

<sup>abcde</sup> Means of treatments along a column with different superscript differed significantly ( $P < 0.05$ ). NC – Negative control, PC – Positive control, FI – feed intake, BWG – Body weight gain, FCR – feed conversion ratio, BW – Body weight, CVu – Uniformity coefficient of variability, ± SEM – standard error of means, P-value – probability level, NC – Negative Control, BD – Basal Diet (Positive Control), BD250 – 250  $\text{mg}\cdot\text{kg}^{-1}$  Beta-glucans, BD375 – 375  $\text{mg}\cdot\text{kg}^{-1}$  Beta-glucans, ECKSe – vitamin E; vitamin C; vitamin K and Selenium.

is presented in Table 3. The AFBW of birds on NC ( $2,011.82 \pm 28.79$  g.bird<sup>-1</sup>) and BD375 + ECKSe ( $1,948.07 \pm 33.84$  g.bird<sup>-1</sup>) were similar but higher ( $P < 0.05$ ) compared to those of birds on other treatment diets. Birds on BD had the least AFBW ( $1,001.49 \pm 17.89$  g.bird<sup>-1</sup>), significantly reduced ( $P < 0.05$ ) compared to birds on other treatment diets. It was noticed that the AFC per bird in birds fed NC (₦580.81  $\pm$  13.86 per bird), BD375 + ECK (₦565.12  $\pm$  17.56 per bird) and BD375 + ECKSe (₦583.43  $\pm$  6.21 per bird) were similar but showed higher variations ( $P < 0.05$ ) from AFC of birds on other diets. The least and significantly reduced ( $P < 0.05$ ) AFC per bird was recorded in birds on BD (₦307.29  $\pm$  10.98 per bird). Average Total Raising Costs- ATRC per bird obtained in birds on NC (₦959.82  $\pm$  13.86 per bird), BD375 + ECK (₦944.13  $\pm$  17.56 per bird), and BD375 + ECKSe (₦962.44  $\pm$  6.21 per bird) were similar but higher ( $P < 0.05$ ), compared to birds fed other diets. The least ( $P < 0.05$ ) ATRC per bird was also obtained in birds fed BD (₦686.31  $\pm$  10.98 per bird) compared to other treatments. The ALWV of birds on NC (₦1,307.68  $\pm$  18.72 per bird) and BD375 + ECKSe (₦1,266.24  $\pm$  21.99 per bird) were comparable and significantly greater ( $P < 0.05$ ) than those of other treatments. The ALWV of birds on BD (₦650.97  $\pm$  11.63 per bird) was the least with significant reduction ( $P < 0.05$ ) compared to birds on other treatment diets, even though the former had the least ATRC. The AMR per bird increased significantly ( $P < 0.05$ ) in birds on NC (₦347.86  $\pm$  20.41 per bird) and BD375 + ECKSe (₦303.80  $\pm$  22.30 per bird), compared to birds fed other treatment diets. Significant ( $P < 0.05$ ) reduction in AMR per bird was recorded in birds on BD (₦35.34  $\pm$  1.01 per bird).

### Effect of varied inclusion levels of beta-glucans, supplemental dietary antioxidants and vitamin K on histological section of liver of broiler chicken

Photomicrographs showing the histological section of liver of broiler chicken fed diets contaminated with aflatoxins but treated with beta-glucans, supplemental dietary antioxidants and vitamin K are presented in Figure 1. Liver microscopic section showed that birds fed NC diet had no observable lesion while birds on BD had gross hepatocellular coagulation and degeneration, with

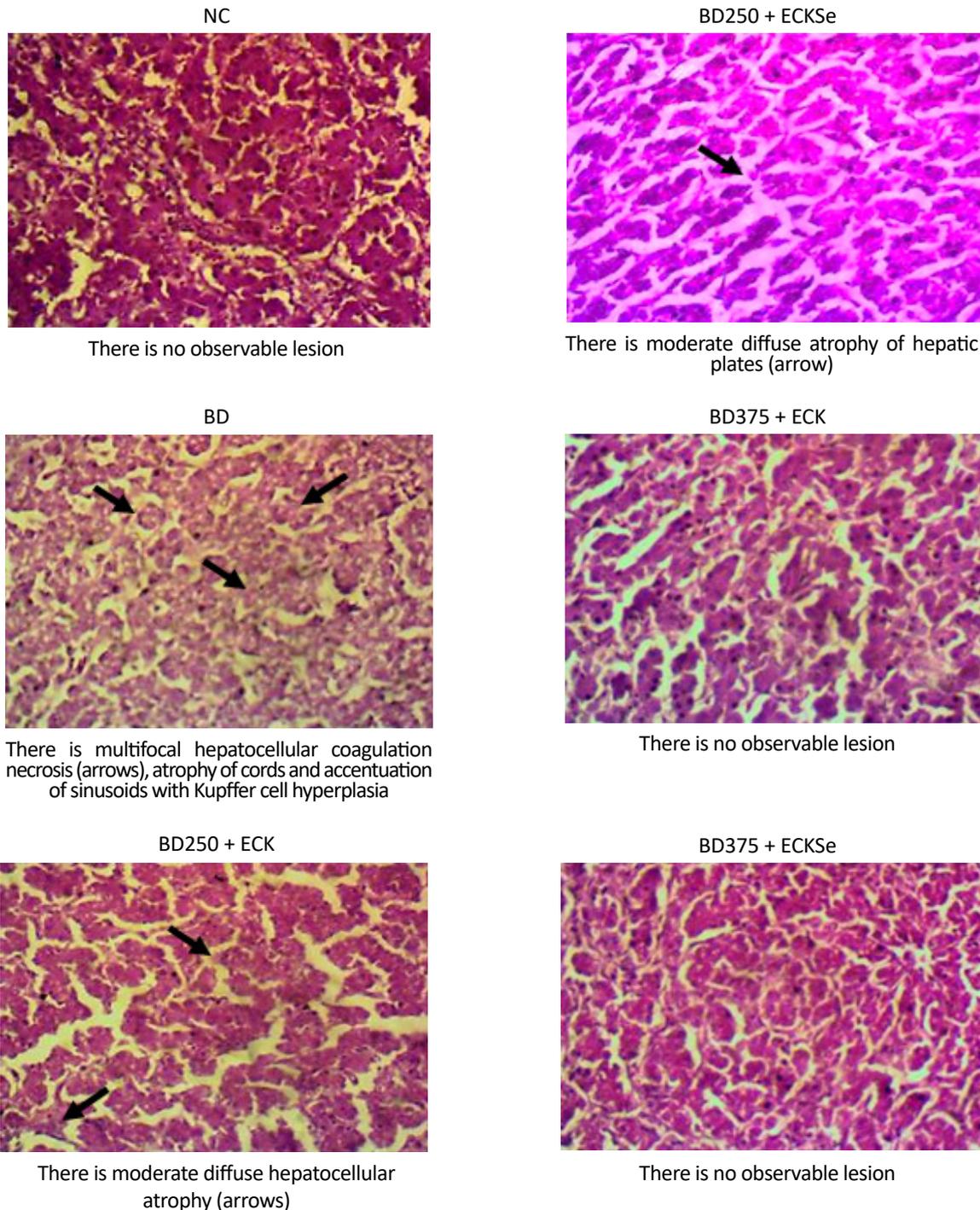
**Table 3: Effect of varied inclusion levels of beta-glucans and supplemental selenium on cost benefit analysis of broiler chicken offered aflatoxin-contaminated poultry feed**

Treatments	Aflatoxins ( $\mu\text{g.kg}^{-1}$ )	Beta-glucans ( $\text{mg.kg}^{-1}$ )	Se ( $\text{mg.kg}^{-1}$ )	AFBW ( $\text{g.bird}^{-1}$ )	AFC per bird (₦)	ATRC per bird (₦)	ALWV per bird (₦)	AMR per bird (₦)
NC	0	0	0.00	$2011.82 \pm 28.79^a$	$580.81 \pm 13.86^a$	$959.82 \pm 13.86^a$	$1307.68 \pm 18.72^a$	$347.86 \pm 20.41^a$
BD (PC)	270	0	0.00	$1001.49 \pm 17.89^e$	$307.29 \pm 10.98^d$	$686.31 \pm 10.98^d$	$650.97 \pm 11.63^e$	$-35.34 \pm 1.01^d$
BD250 + ECK	270	250	0.00	$1275.22 \pm 63.90^d$	$402.80 \pm 5.25^c$	$781.81 \pm 5.25^c$	$828.89 \pm 41.54^d$	$47.08 \pm 39.32^c$
BD250 + ECKSe	270	250	0.30	$1425.37 \pm 26.93^c$	$437.98 \pm 7.98^b$	$816.99 \pm 7.98^b$	$926.49 \pm 17.51^c$	$109.50 \pm 20.90^{bc}$
BD375 + ECK	270	375	0.00	$1693.73 \pm 109.09^b$	$565.12 \pm 17.56^a$	$944.13 \pm 17.56^a$	$1100.93 \pm 70.91^b$	$156.80 \pm 73.45^b$
BD375 + ECKSe	270	375	0.30	$1948.07 \pm 33.84^a$	$583.43 \pm 6.21^a$	$962.44 \pm 6.21^a$	$1266.24 \pm 21.99^a$	$303.80 \pm 22.30^a$
$\pm$ SEM				32.49	9.46	9.46	21.12	21.46
P-value				<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

<sup>abcde</sup>Treatment means along the same column having different superscripts are significantly different ( $P < 0.05$ ).  $\pm$  SEM – Standard error of mean, P-value – probability level, AFBW – Average Final Bodyweight, AFC – Average Feed Cost, ATRC – Average Total raising cost, ALWV – Average liveweight value, AMR – Average Marginal return, NC – Negative Control, BD – Basal Diet (Positive Control), BD250 – 250 mg.kg<sup>-1</sup> Beta-glucans, BD375 – 375 mg.kg<sup>-1</sup> Beta-glucans, ECKSe – vitamin E; vitamin C; vitamin K and Selenium, Nigerian naira- ₦, equivalent to 0.0024 USD.

atrophy and sinusoids accentuation, coupled with hyperplasia of the Kupffer cells as indicated with the arrows. However, birds on diets that had 375 ppm beta-glucans with or without supplemental selenium

(BD375 + ECKSe and BD375 + ECK respectively) resulted in improvement in the hepatic tissues' histology with no observable lesion as seen in birds on NC. Addition of 250 ppm of beta-glucans with



**Figure 1. Histological section of liver of broilers fed aflatoxin-contaminated poultry feed Magnification = x400 for each slide; Stain = Haematoxylin and Eosin**

(BD250 + ECKSe) or without (BD250 + ECK) selenium supplementation had moderate hepatocellular degeneration (arrows). The results of histology of liver samples from birds fed BD250 + ECK to BD375 + ECKSe confirmed positive effect of beta-glucans in preventing extensive liver damage as seen in birds on BD.

## DISCUSSION

The reduction in ALT values observed in birds on BD250 + ECK to BD375 + ECKSe (the mitigated diets), whose values were in-between what was recorded in birds fed uncontaminated and the unmitigated diets, showed the reduction of injury to the liver of birds in the mitigated diets. Elevation in serum ALP value may arise from obstruction of biliary ducts, as reported by Peles *et al.*, (2019) during aflatoxins poisoning. Addition of beta-glucans, supplemental antioxidants and vitamin K resulted in reduced ALP values similar to those of birds fed NC diet, which indicated the reduction in liver damage. The reduced ALP values showed that birds feeding on contaminated but mitigated diets containing vitamin K all had low level of ALP. This may be a result of reduced biliary duct haemorrhage due to the presence of vitamin K, which is required to bypass the inhibitory effect of aflatoxins on vitamin K epoxide reductase (Shearer and Newman, 2008). Reduction in ALP values following the addition of the mitigating substances may be considered as one of the outcomes of effective amelioration of the anticoagulation effect of aflatoxins in broiler chickens.

ROS including free radicals (FR), such as hydroxyl anion radical (OH<sup>•</sup>) and non-radical such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) productions are characteristic of microsomal enzymes – cytochrome P450 metabolic by-products (Reed *et al.*, 2011). Stimulation of hepatic membrane lipid peroxidation by FR/ROS is one of the mechanisms of aflatoxins toxicity (Omar, 2013). Malondialdehyde is one of the secondary end products of lipid peroxidation and a common and reliable biomarker for lipid peroxidation activity (Esterbauer and Cheeseman, 1990). Elevated plasma malondialdehyde as observed in birds fed unmitigated basal diet, could indicate high lipid peroxidation activity in the liver of birds

fed BD. Addition of beta-glucans, supplemental antioxidants and vitamin K reduced plasma malondialdehyde, indicating reduction in lipid peroxidation activity and also a reduction in oxidative stress (Giera *et al.*, 2012). Birds fed BD375 + ECKSe had marked reduction of more than 50% in plasma malondialdehyde compared to birds on unmitigated diet. The marked reduction in plasma malondialdehyde values of birds on higher beta-glucans levels compared to birds on unmitigated group, was one of effective means of counteracting aflatoxins' deleterious effects in broiler chickens.

Birds fed unmitigated contaminated diet had higher oxidised glutathione values. Elevated oxidised glutathione level was greatly reduced in all four treated diets supplemented with the mitigating substances. Earlier, it was reported (Adeogun *et al.*, 2021a) that combinations of supplemental antioxidants and vitamin K alone as a counteracting measure could not markedly reduce oxidised glutathione values in birds fed aflatoxin-contaminated diets. The reduction in absorbed aflatoxins in all the four mitigated diets by beta-glucans resulted in marked reduction in oxidised glutathione values due to reduced H<sub>2</sub>O<sub>2</sub> from aflatoxin metabolism and reduction in glutathione usage. Oxidative stress situation arises when there is an imbalance between antioxidants in the body and the prevailing oxidants. Oxidative stress can be measured by determining the ratio between reduced glutathione (GSH) and the oxidised (GSSG) glutathione (Pastore *et al.*, 2001). When the balance is in favour of GSSG, there is oxidative stress situation and when it is in favour of GSH, oxidative stability could be inferred. Results from Table 2 demonstrate that effective reduction of absorbed aflatoxins is a vital prerequisite to any other complementary strategy. Birds on 250 mg.kg<sup>-1</sup> beta-glucans diets had similar GSH:GSSG ratios while birds on 375 mg.kg<sup>-1</sup> beta-glucans diets had higher but similar values. Higher GSH:GSSG ratio similar to those obtained in birds fed uncontaminated diet indicated effective mitigation against oxidative stress effect.

Vitamin C was co-included with vitamin E, to enhance the latter antioxidant capacity through synergism, by acting as a co-antioxidant. This step enhanced the recycling of  $\alpha$ -tocopheryl radical generated during the neutralisation of free radical or ROS produced during aflatoxin metabolism in

the liver, back to active vitamin E ( $\alpha$ -tocopherol). Addition of vitamin C will, therefore prevents early depletion of vitamin E, and enhances the prevention of hepatic microsomal phospholipids peroxidation. Selenium is an indispensable cofactor in the activity of glutathione peroxidase, the enzyme endowed by nature to neutralise hydrogen peroxide ( $H_2O_2$ ) and peroxy radical ( $ROO\cdot$ ). The addition of selenium into the diet may result in reduction of peroxidative damage to hepatic cells membrane, maintaining redox balance and preventing oxidative stress situation. This was also evident in the levels of malondialdehyde and the GSH:GSSG ratios recorded in the current study.

Vitamin K (an anti-haemorrhagic agent) was included into the diets to prevent or minimise the anticoagulant ability of aflatoxins, in view of the structural similarity between aflatoxins, 4-hydroxycoumarin and synthetic dicoumarol (Bababunmi, 1989). Aflatoxins react in a similar manner to 4-hydroxycoumarin and dicoumarol in binding with the enzyme vitamin K epoxide reductase, which converts vitamin K epoxide back to vitamin K (the quinone form), within the vitamin K cycle, following the post translational modification and production of "gla proteins" (Shearer and Newman, 2008). The gla protein is a prerequisite in blood clotting factor synthesis. Despite the anticipated blockage of vitamin K recycling by aflatoxins, supplementation of vitamin K may have ensured "gla protein" production via consistent supply of dietary vitamin K. This resulted in effective mitigation of the anti-coagulant effect of aflatoxins. The reduction in the alkaline phosphatase level in the current study evidences for the improvement in the mitigation of the haemorrhagic potential of aflatoxins (Peles *et al.*, 2019). The reduction in serum alkaline phosphatase, malondialdehyde and higher reduced glutathione to oxidised glutathione ratio, resulted in better performance indices in birds on the contaminated but mitigated diets over those of birds fed unmitigated diet.

Birds fed diet BD375 + ECKSe had comparable performance to birds fed uncontaminated diet, except for mortality. The mortality of birds showed, however, a significant improvement over that of birds on the unmitigated diet. Aflatoxin suppresses growth by impairing vital metabolic processes in the body. ROS such as  $H_2O_2$ , which may be produced

during aflatoxins metabolism, is permeable to cell membranes (Halliwell and Gutteridge, 1989) and when produced in excess of the capability of catalase and glutathione peroxidase to neutralise, it can obstruct the glycolytic pathway. Excessive  $H_2O_2$  has been reported to inhibit glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Mullarky and Cantley, 2015), a rate limiting enzyme that catalyses the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Reduction in aflatoxin absorption with beta-glucans may result in reduced  $H_2O_2$  production, as earlier reported by Adeogun *et al.* (2021b).

Addition of beta-glucans, supplemental antioxidants and vitamin K in broilers diet contaminated with aflatoxins helped in counteracting these impairments of vital metabolic processes by ensuring that reduced glutathione is not depleted, and also, by allowing easy production of NADPH. Molavian *et al.* (2016) reported that excessive accumulation of  $H_2O_2$  will result in cell death. This may partly be the reason for high mortality recorded in birds on the unmitigated diet and that addition of the mitigating substances were possibly the reason for the reduced mortality in birds fed 375 mg.kg<sup>-1</sup> beta-glucans diets.

In addition, the higher levels of reduced glutathione above that of oxidised glutathione in all treatment diets supplemented with beta-glucans, antioxidants (with or without selenium) and vitamin K were indications that the level of  $H_2O_2$  was within physiological tolerance limit or the cell sensitivity concentration (CSC) level (Molavian *et al.*, 2016), leading to reduced mortality. Of the numerous ways in which excessive ROS generated from aflatoxins metabolism retard growth, the effect on the Krebs or TCA cycle is worthy of mention briefly. Excessive  $H_2O_2$  production arising from high level of superoxide anion ( $O_2^-$ ) generation during hepatic microsomal aflatoxins metabolism, inhibits three of the TCA cycle enzymes (Tretter and Adam-Vizi, 2000), notably: aconitase,  $\alpha$ -ketoglutarate dehydrogenase and succinate dehydrogenase. These enzymes catalyze directly or indirectly reaction steps that lead to the production of isocitrate,  $\alpha$ -ketoglutarate, succinyl CoA, malate and oxaloacetate. These intermediate products of the TCA cycle though produced in the mitochondria, are capable of crossing the mitochondria membrane into the cytosol, where they can undergo

one or more transamination reactions to produce their corresponding amino acids (Campbell and Farrell, 2008), the building blocks in protein synthesis. Controlling excessive production of ROS such as  $H_2O_2$  in broiler chickens consuming aflatoxin-contaminated diet using beta-glucans and supplemental antioxidants will mitigate the inhibition of these enzymes and the obstruction in the activities of the TCA cycle.

The adverse effects of aflatoxins are multifaceted when ingested and it goes beyond obstructing carbohydrate metabolism. Obstruction of the biliary tract will impede bile flow into the duodenal region of the GIT, impairing micelle formation, hence, fat and fat-soluble vitamins absorption will be affected. Consequently, the utilization of dietary fat will also be affected. Hyperplasia of the biliary duct of birds fed mitigated diets was counteracted with consistent dietary vitamin K supply, by making sure that even though aflatoxins bind to and inhibit one of the apoenzymes (vitamin K epoxide reductase) required in the blood clotting factors formation (the gla proteins), the process goes on through an alternative pathway (Shearer and Newman, 2008). Therefore, minimising the haemorrhagic effect of aflatoxins on the hepatic and biliary tract cells was evident in the ALP levels recorded in birds fed contaminated but mitigated diets. The overall effect of the added mitigating agents on these biochemical reactions resulted in no observable lesions in the liver of birds on the treated diets, leading to a reduction in mortality.

## CONCLUSION

Addition of beta-glucans, supplemental dietary antioxidants (vitamins E, C and selenium) and vitamin K resulted in improved performance of birds fed diets contaminated with aflatoxins, similar to birds fed uncontaminated diet. Liver deterioration caused by aflatoxins poisoning, leading to marked increase in mortality was also prevented. The current study demonstrates that vitamins C, E and K along with selenium which are normal components of broiler chickens' vitamins and minerals premix can be used to mitigate the adverse effects of dietary aflatoxins when supplemented in the right proportions. This study suggests that growth retardation by dietary

aflatoxins in broiler chickens could be effectively mitigated by supplementation of the diet with yeast beta-glucans at  $375 \text{ mg.kg}^{-1}$  combined with 200 mg of vitamin E, 250 mg of vitamin C, 0.3 mg of selenium and 3 mg of vitamin K per kg of feed.

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Writing-review and editing: Ewuola, E. O., Abu, O. A.

Project administration: Adeogun, J. B., Abu, O. A., Ewuola, E. O.

All authors have read and agreed to the published version of the manuscript.

## INFORMED CONSENT STATEMENT

Not applicable.

## DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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