

Dietary glycine-coated phytogenic Ag/TiO₂ nanohybrids attenuate aflatoxin B₁ induced physiological perturbations without impacting performance, meat quality, and carcass-visceral traits of finisher broilers

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Abstract

This study investigated the ameliorative effects of dietary glycine-coated green Ag/TiO₂ hybrid nanoparticles (Gly-G-Nano-Ag/TiO₂) on aflatoxin B₁ (AFB₁) toxicity in finisher broilers. One hundred and twenty (120) 21-day old Cobb 500 male chicks were offered in a completely randomized design 3 treatment diets [i.e., Control diet without AFB₁ (-AF); and Diets with 0.04 mg/kg AFB₁ (+AF); and 0.04 mg/kg AFB₁ plus 25 mg/kg Gly-G-

Nano-Ag/TiO₂ (+AF+Gly_n)] each with 8 replicate pens of 5 for 21 days. Data on performance, carcass traits, hemato-biochemistry, and meat quality were collected and statistically analysed. Results showed no effect of diet on growth performance, carcass traits, internal organs, meat quality, and most haemato-biochemistry variables ($P > 0.05$). However, dietary AFB₁ decreased white blood cells and serum albumin, both of which were restored by Gly-G-Nano-Ag/TiO₂ ($P < 0.05$ and $P < 0.001$, respectively). Also, it decreased blood lymphocytes, though this response was however not ameliorated by the green bimetallic nano-hybrids ($P < 0.001$). In contrast, both AFB₁ and Gly-G-Nano-Ag/TiO₂ increased blood heterophils and platelets ($P < 0.001$). In conclusion, dietary AFB₁ and Gly-G-Nano-Ag/TiO₂ induced no effects on growth performance, carcass characteristics, internal organs, meat quality, and most haemato-biochemistry variables but increased blood heterophils and platelets of broilers as the aflatoxin decreased their blood lymphocytes. The green nanoparticles attenuated detrimental effects of AFB₁ on white blood cells and serum albumin.

Keywords: Broiler, aflatoxicosis, nanoparticles, health status, nutritional status

Introduction

Broiler chickens are the most abundant meat birds globally, with their consumption growing faster than any other meat type, particularly in developing countries (FAO, 2018; OECD, 2022). In South Africa, the African continent's largest producer of the birds, their production dominates the agricultural sector, and they are the main dietary protein supplier surpassing all other animal-derived proteins combined. Also, their meat contains significant amounts of highly digestible proteins with a low content of saturated fats (Christensen et al., 2018). Moreover, the broilers are indeed an affordable human dietary protein source most ideal for ensuring food security for underprivileged households (Rodic et al., 2011 and Dana, 2019). However, their production and quality of their meat are increasingly threatened by feed contaminants including mycotoxins. In this regard, climate change-associated elevated

environmental temperatures with concomitant droughts, greenhouse gases, and humidity especially in tropical/subtropical climates enhance the proliferation of toxigenic fungi and the risk of contamination of plant-derived feed resources such as maize and seed cakes, the predominant energy and protein sources for broiler chicken diets, with mycotoxins primarily the aflatoxins (Battilani et al., 2016; Casu et al., 2024).

Aflatoxins are produced by fungi belonging to the *Aspergillus* genus with *A. flavus* being the most threatening species that produces aflatoxin B₁ (AFB₁), the mutagen, immunotoxin, teratogen, and highly problematic Group 1 carcinogen with capacity to cause human liver cancer (IARC, 1987; Ostry et al., 2017; Yilmaz et al., 2017). The *Aspergillus* fungi commonly infest crops meant for human and animal nutrition during growth, harvesting, storage, transportation, and processing (Bryden, 2007). Hence, human consumption of

aflatoxin-contaminated maize has led to hundreds of human death cases in Africa (Lewis et al., 2005). Also, dietary exposure of broiler chickens to AFB₁, mainly derived from maize and oilseed cakes (meals) used as energy and protein sources, leads to intestinal absorption of the aflatoxin (Grenier & Applegate, 2013). Upon absorption, the generally non-toxic AFB₁ is translocated into the liver where it is metabolized by the cytochrome P450 enzymes into the highly toxic and carcinogenic AFB₁-8,9-epoxide (AFBO) radical (Gallagher et al., 1994, 1996; Kuilman et al., 2000). It is through this mechanism that AFB₁ induces toxicity primarily mediated by generation of reactive oxygen species (ROS) and free radicals that induce oxidative stress and concomitant damage to cell membranes and macromolecules (Cao et al., 2022). The toxicity is manifested as compromised chicken body weight gain (BWG), feed intake (FI), feed conversion efficiency (FCE), intestinal integrity, immune function, and meat quality including deposition of AFB₁ residues in edible tissues (Asim et al., 1990; Bintvihok et al., 2002; Hernández-Ramírez et al., 2021; Sarker et al., 2021). These deleterious effects of AFB₁ therefore accentuate the need for an innovative strategy to resolve the problem of toxicity of the aflatoxin in broilers.

Generally, toxicity of AFB₁ and other feed-born mycotoxins has conventionally been remedied through dietary incorporation of adsorbent feed additives including clay particles (bentonites and zeolites) (Deng et al., 2023). These additives however suffer

the disadvantage of also binding to dietary minerals, vitamins and amino acids (Zhu et al., 2016). Consequently, symptoms of vitamin deficiency and decreased serum levels of zinc, copper and manganese in contrast to increased levels of aluminium would occur in chickens fed diets incorporated with bentonites or zeolites (Deng et al., 2023). This circumstance would thus necessitate dietary supplementation with increased inclusion levels of the deficient micronutrients by an average of 20% (Brown et al., 2014; Bhatti et al., 2018), leading to increased production costs. Another disadvantage with adsorbent feed additives is that their effectiveness only ranges from 20 to 80% and is decreased by gastric juices (Horky et al., 2018) as well as by feed structure, moisture content and oxygen availability (Vila-Donat and Marin 2018; Jampilek and Kralova, 2020). Furthermore, metallic oxide type of adsorbent feed additives such as graphene oxide have previously been used but they also exhibited the disadvantage of adsorbing minerals including copper, magnesium and zinc and slightly inhibiting the digestive enzymes α -amylase and trypsin (Horky et al., 2020).

As a more effective and sustainable solution, this study proposes an innovative nanotechnologically-delivered glycine strategy based on cellular metabolism of AFB₁. In this regard, AFB₁ detoxification involves glutathione-S-transferase-mediated conjugation of AFBO to glutathione (GSH) via the mercapturic acid pathway leading to formation of

AFBO-GSH conjugates (Bryden, 2012) that are excreted as non-toxic mercapturic acid products (AFB-NAC) in bile and urine (Wirth and Thorgeirsson, 1976). As a precursor for the synthesis of glutathione (McCarty et al., 2018), it is hypothesized herein that dietary administration of glycine to broiler chickens would bolster their cellular GSH levels and empower their ability to detoxify AFB₁-derived AFBO. Indeed, many studies have demonstrated dietary glycine supplementation to improve antioxidant capacity in chickens (Xie et al., 2016; Li et al., 2009; Wang et al., 2013) through enhancement of GSH synthesis (Wang et al., 2017; Gheller et al., 2021). Also, one study demonstrated that dietary supply of glycine improves liver GSH and activity of antioxidant enzymes catalase and superoxide dismutase whilst ameliorating the toxicity of neonicotinoid insecticide imidacloprid in broiler chickens (Abbas et al., 2021).

Notwithstanding, aside the hydrophilicity of amino acids which renders them unable to freely translocate through cell membranes (Bhutia et al., 2015), dietary supply of glycine to animals is limited by substantial catabolism (50%) of the amino acid within the small intestinal mucosa (Stoll et al., 1998), which compromises the efficiency of its utilization. For this reason, there is a need for an innovative strategy such as nanotechnology to protect glycine from enteric catabolism and to enhance its delivery to cells where GSH synthesis takes place. Indeed, amino acid transporter targeted nanocarriers have

been shown to enhance amino acid uptake in various biological systems (Zhang et al., 2020). In particular, amino acid-coated nanoparticles such as nano-metal-amino acid chelates are an efficient strategy for enhancing intestinal absorption and delivery into poultry of antioxidant precursor amino acids (Rahmatollah et al., 2018) that bolster the antioxidant status of target cells thus protecting them against free radicals and ROS (Lafleur et al., 1980; Bogunia-Kubik and Sugisaka, 2002; Nel et al., 2006).

Notwithstanding, no studies hitherto have employed green Ag/TiO₂ bimetallic nanoparticles to facilitate intestinal uptake of glycine into broiler chickens subjected to aflatoxicosis. Hybrid bimetallic nanoparticles possess much more reactive properties than their monometallic counterparts (Adeyemi et al., 2019, 2022) particularly when silver (Ag) is used as dopant for other metals and metal oxides (Elemike et al., 2019). The combined reactive properties of doped bimetallic alloy nanoparticles offer the nanomaterials more advantages over ordinary monometallic nanoparticles (Mohl et al., 2011). However, no studies have hitherto investigated ameliorative effects of *Mondia whitei* extract-fabricated glycine-coated green Ag/TiO₂ hybrid nanoparticles (Gly-G-Nano-Ag/TiO₂) against the toxicity of AFB₁ in broilers. Widely distributed in tropical Africa from Guinea through Cameroon to East and Southern Africa, *M. whitei* (*Periplocaceae*) is a woody climbing plant with large tuberous roots that are commonly used as spices, aphrodisiacs

and for treatment of various ailments (e.g., urinary tract infections, jaundice, headache, diarrhoea, and others) (Adjanohoun et al., 1996; Noumi et al., 1998). The plant is naturally endowed with a plethora of phytochemicals including alkaloids, carotenoids, 5-chloropropacin, heptacosane, 9-hexacosene, 2-hexen-1-ol, hydrogen cyanide, 2-hydroxy-4-methoxybenzaldehyde, isovanillin, (-)-loliolide), oxalate, para-pentylphenylbenzoate, phenolics, phytate, propacin, reducing sugars, saponins, steroids, tannins, and triterpenes (Deeh et al., 2024) that may serve as stabilisers and reducing agents in the biosynthesis of Gly-G-Nano-Ag/TiO₂. Therefore, the objective of this study was to investigate the efficacy of *M. whitei* root extract-fabricated Gly-G-Nano-Ag/TiO₂ against AFB₁ toxicity in terms of growth performance, carcass characteristics, internal organs, haemato-biochemistry, and meat quality in finisher broilers.

Materials and Methods

Ethical Approval and Study Site

The study protocol was approved by Animal Production Science Research Ethics Committee (NWU-00809-23-A5) of North-West University (NWU). This study was carried out at the NWU experimental farm, Molelwane (GPS coordinates: 25° 40.459'S, 26° 10.563'E), located in the Mahikeng Local Municipality, North-West Province, South Africa.

Sourcing of Experimental Materials

The feed ingredients were purchased from SimpleGrow Agric Services in Centurion, Gauteng Province, South Africa. *Mondia whitei* foliage (leaves and vines) was collected from KwaDlangezwa area in KwaZulu-Natal province, South Africa. Glycine, silver nitrate (AgNO₃), titanium butoxide (Ti(OBu)₄), NaOH, and all other chemicals were purchased from Merck (Pty) Ltd., South Africa. Cobb 500 broiler chicks were purchased from Superior Chicks (Pty) Ltd (Farm Grootfontein, Pretoria, South Africa).

Green Synthesis of Glycine-Coated Green Ag/TiO₂ Hybrid Nanoparticles

The nanoparticles were green synthesized using the fresh extract of *M. whitei* foliage according to the method as described by Elemike et al. (2019), with slight modifications. Approximately 50 mL of the foliage extract was added to 250 mL of 1 mM of the respective metal salts (AgNO₃ and Ti(OBu)₄) and stirred on a hot plate for 1 h at 85 °C using a magnetic stirrer. Similarly, for the TiO₂ nanoparticles, 100 mL of foliage extract was added to a flask with 250 mL of 1 mM of Ti(OBu)₄ solution and stirred for 1 h at 85 °C. For the synthesis of Ag/TiO₂ bimetallic nanoparticles, 250 mL of 1 mM of AgNO₃ was mixed with 50 mL foliage extract and stirred for 30 min at 85 °C followed by addition of a mixture of 250 mL of 1 mM of Ti(OBu)₄ and 50 mL foliage extract. These reactions were monitored periodically for changes in colour using UV-vis spectrophotometry. The solution was then centrifuged at 4500 rpm for 10 min and the precipitate rinsed with a

mixture of water/ethanol 3 times to remove any impurities. The product was then transferred to a crucible and left overnight to dry, after which it was calcinated at 450 °C for 2 h. The glycine-coated Ag/TiO₂ nanoparticles were produced by mixing the synthesized Ag/TiO₂ nanoparticles with glycine in a ratio of 1:1 (w/w) in a colloidal mixer to produce a eutectic melt that hardened to a white solid. The solid material was then ground with a pestle and mortar to a fine powder to produce Gly-G-Nano-Ag/TiO₂.

Production of Aflatoxin B₁

Culturing of Fungus

Wheat flour was used for culturing the fungus following a method described by Makun et al. (2011), with few modifications. Pure mycelium of *Aspergillus flavus* was cultured on Malt Extract Agar (MEA). To prepare the culture medium, 25 g of MEA were weighed and added into 500 mL of distilled and the mixture was thoroughly mixed in a conical flask. The mixture was then autoclaved at 100 °C for 1 h and cooled at room temperature. After cooling, the media was poured into petri dishes and incubated at 28 °C for 7 days. For wheat flour medium preparation, 100 g of flour was weighed, covered with cotton and foil, and autoclaved at 100 °C for 1 h. After cooling it for 20 min, 1 g of the media was weighed and mixed with 12 % distilled water. The suspension was then inoculated in the flask containing the flour substrate and the flask was incubated at 28 °C as a static culture for 21 days.

Extraction of Aflatoxin B₁

Extraction of AFB₁ from the flour fungal culture material was performed following the method developed by Abdel-Monem et al. (2015). Briefly, 25g of the sample and 2.5 g of sodium chloride (NaCl) was weighed and mixed with 100 mL of solvent prepared using 80 % methanol and 20 % distilled water. The mixture was stirred for 5 min and then filtered using a Whatman filter paper, followed by Solid Phase Extraction (SPE) through packing the column with silica gel (Easy-extract aflatoxins, r-Biopharm Rhône LTD, Glasgow, Scotland, UK). Thereafter, 2 mL of the filtrate was added to 14 mL of phosphate buffer saline (PBS), mixed thoroughly using a vortex, and applied into the columns. After rinsing with 20 mL PBS and 2 mL of 99% methanol, the mixture was analysed using High Performance Liquid Chromatography (HPLC) (Shimadzu FCV-20H2; Kyoto, Japan) equipped with a fluorescence detector (a coring cell (CoBrA cell) (Dr Weber Consulting, Mannheim, Germany).

Experimental Design, Diets, and Bird Management

Day-old Cobb 500 chicks were initially fed commercial starter (day 1-14) and grower (day 15-21) diets. When they were 21 days old, they were sexed, and 120 males of similar body weight (730.33 ± 4.93) were randomly allocated to 24 pens [size: ($h \times b \times l$) 1.8 m × 1.5 m × 1.5 m]. The pens were then randomly allocated to 3 experimental finisher diets [a control diet without AFB₁ (-AF), a diet containing 0.04 mg/kg

AFB₁ (+AF), and a diet containing 0.04 mg/kg AFB₁ and 25 mg/kg Gly-G-Nano-Ag/TiO₂ (+AF + Gly_n) in a completely randomised design (CRD). The diets were formulated according to National Research Council (NRC, 1994) guidelines to meet or exceed the nutrient requirements of broiler chickens at finisher phase (Table 1). Each dietary treatment was replicated 8

times with 5 birds each. The pen was the experimental unit. The feeding trial was carried out for 21 days (d21 – 42) in a broiler house in a deep litre system, with temperatures maintained between 23 and 31 °C throughout the feeding trial. Artificial lighting was provided using electric lights. Fresh clean water and feed were provided *ad libitum* for the whole duration of the experiment.

Table 1. Ingredient and nutritional composition (% as fed basis, unless stated otherwise) of experimental broiler finisher diets.

Ingredient	Experimental diets		
	-AF	+AF	+AF+Gly _n
Yellow maize	64.31	64.31	64.31
Soyabean meal (47% CP)	17.97	17.97	17.97
Full fat soyabean meal	14.02	14.02	14.02
Limestone powder	1.28	1.28	1.28
Mono-dicalcium phosphate	1.35	1.35	1.35
L-Lysine HCL	0.10	0.10	0.10
DL-Methionine	0.20	0.20	0.20
L-Threonine	0.03	0.03	0.03
Salt	0.31	0.31	0.31
Salinomycin	0.05	0.05	0.05
Zinc bacitracin	0.05	0.05	0.05
Sodium bicarbonate	0.18	0.18	0.18
Vitamin-mineral premix*	0.25	0.25	0.25
Aflatoxin B ₁ (mg/kg)	0.00	0.04	0.04
Green Nano-Gly-Ag/TiO ₂ (mg/kg)	0.00	0.00	25
Total	100	100	100
<i>Nutritional composition, aflatoxin B₁ and its metabolites</i>			
Dr matter	87.90	87.92	87.94
Crude protein	18.03	18.01	18.04
Crude fibre	3.44	3.45	3.42
Ether extract	5.36	5.38	5.34
Ash	5.33	5.38	5.37
Metabolizable energy (MJ/kg)	12.0	12.0	12.0
Calcium	0.80	0.81	0.83
Phosphorus	0.35	0.36	0.36
Ca:P ratio	2.29	2.25	2.31
Lysine	1.03	1.04	1.05
Methionine	0.47	0.47	0.46
Threonine	0.73	0.72	0.71
Valine	0.87	0.86	0.87
Aflatoxin B ₁	0.06	0.02	0.01
Aflatoxin G ₂	0.12	0.08	0.09
Aflatoxin G ₁	0.14	0.25	0.10

Aflatoxin B ₂	0.01	0.03	0.02
*Vitamin-mineral premix contained: 0.12 g biotin; 30 mg niacin; 0.7 mg folic acid; 10 mg pantothenic acid; 11,000 IU vitamin A; 4.5 mg vitamin B ₂ ; 2.5 mg vitamin B ₁ ; 1 mg vitamin B ₆ ; 2500 IU vitamin D ₃ ; 2.0 mg vitamin K ₃ ; 25 IU vitamin E; 100 mg magnesium sulphate; 8.0 mg copper sulphate; 80 mg ferrous sulphate; 79 mg zinc sulphate; 0.34 mg potassium iodine; 0.25 mg sodium selenite. AF = aflatoxin B ₁ ; Gly _n = glycine-coated green Ag/TiO ₂ hybrid nanoparticles.			

Aflatoxin Analysis of Experimental Diets

Samples of experimental diets were analysed for AFB₁ and its metabolites (Table1) using a HPLC (Shimadzu FCV-20H2; Kyoto, Japan) equipped with a fluorescence detector (a coring cell (CoBrA cell) (Dr Weber Consulting, Mannheim, Germany) according to the procedure of Zaghini et al. (2005), however with some modifications. The feed samples (2 g) were weighed and placed into a beaker. A mixture containing 2 mL of 20% N-hexane and 4 mL of methanol was added into the feed samples and thoroughly mixed. The mixture was centrifuged at 3000 rpm for 30 min, filtered using a Whatman filter paper, and the eluate used for solid phase extraction (SPE) using a column with silica gel (Easy-extract aflatoxins, r-Biopharm Rhône LTD, Glasgow, Scotland, UK). Thereafter, the solution compositing 20 mL PBS and 2 mL of 99% absolute methanol was added to the eluate and vortexed. It was added to 500 mL of acetonitrile/water solution (50:50 vol: vol) (HPLC grade). The purified and derivatized samples were injected into a liquid chromatograph consisting of a solvent delivery system and an autosampler fitted with a 20-μL loop (Beckman) coupled to a fluorometric detector (Jasco 821 FP) with wavelengths set at 361 nm for

excitation and 430 nm for emission. The samples were chromatographed on a C18 LUNA column (5 μm and 150 × 4.6 mm; Phenomenex) with a C18 security guard column (5 μm and 45 × 4.6 mm; Phenomenex). The mobile phase (flow rate of 0.8 ml/min) was water/acetonitrile/methanol solution (66:25.2:8.8 vol; HPLC grade). The AFB₁ and metabolite reference working standard solutions were prepared at different concentrations to generate a reference curve. Aliquots were derivatized with trifluoroacetic acid 10 (analytical grade) and injected into the liquid chromatograph as the unknown samples (reference curve).

Growth Performance Measurements

The FI was calculated daily by subtracting the weight of leftover feed from the weight of feed offered and dividing the difference by the total number of birds per pen. Body weight was first measured on d21 to get the initial weight and weekly thereafter. Then BWG per bird was calculated by subtracting the previous body weight from the present body weight and dividing by the number of birds per pen. Feed conversion efficiency (FCE) was computed by dividing the weekly BWG by the weekly FI.

Carcass Traits and Visceral Organs

Following 12 h of feed withdrawal at day 42, 4 birds were randomly selected from each replicate pen, electrically stunned, bled, and de-feathered. A total of 32 birds per treatment (96 birds overall) were evaluated for carcass characteristics. The hot carcass weight (HCW) was measured by weighing their eviscerated carcasses within 45 min of slaughter whilst cold carcass weight (CCW) was measured after chilling the carcasses at 4 °C for 24 h. Also, carcass traits (breast, wing, thigh, and drumstick) of the left side were determined by removing each body part from the whole carcass and individually weighing it using an electronic scale. To minimize variation associated with carcass processing, all dissections and tissue separations were performed by the same trained personnel using standardized anatomical landmarks and cutting procedures throughout the study. The lengths and weights of the visceral organs (liver, gizzard, proventriculus, spleen, duodenum, jejunum, ileum, caecum, and colon) were also measured, respectively, using a tape measure mounted on a weighing board and a digital scale. The weights of the visceral organs were expressed as a percentage of HCW while the weight of the carcass traits was expressed as percentage of CCW.

Haemato-biochemistry Parameters

On day 42, 5 a 21-gauge needle with a 5 mL syringe was used to collect blood from the wing vein of 2 birds per pen (16 per treatment). The blood (1 mL)

was placed in purple-top EDTA-coated vacutainer tubes and analysed for haematology using a computerised IDEXX LaserCyte Hematology Analyzer (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa). Also, some of the blood (4 mL) was placed into red top Vacuette® Serum Clot Activator tubes without EDTA (Greiner Bio-One, GmbH, Frickenhausen, Germany) for the analysis of serum biochemistry using a computerised IDEXX Catalyst One Analyser (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa).

Meat Quality Analysis

Following slaughter, scalding, defeathering, and evisceration, meat quality measurements were performed on the breast muscle. The meat pH and temperature of the breast muscle were measured at an interval of 45 min post-slaughter and 24 h after chilling 4 °C) using a portable fibre-optic pH-temperature metre (Hanna instrument, H198163, Romania). Breast muscle colour (L^* - lightness, a^* - redness, and b^* - yellowness) was measured using a calibrated Konica Minolta colour guide (Narich (Pty) Ltd, Cape Town, RSA). Water-holding capacity (WHC), drip loss, and cooking loss were determined according to the procedures described by (Mthana and Mthiyane 2024). For WHC and drip loss determination, 20 g breast muscle samples were excised. Water-holding capacity was measured using the filter-paper press method under a pressure of 60 kg for 5 min. For drip loss determination, samples were individually suspended in sample bottles and stored at 4°C for 72 h

without contacting the sides or bottom of the containers. For cooking loss determination, breast muscle samples (40–50 g) collected 24 h post-slaughter were sealed in watertight plastic bags and cooked in a water bath at 85°C for 45 min. After cooling to room temperature, the samples were reweighed and cooking loss was expressed as the percentage weight loss during cooking.

Statistical Analysis

All data were analysed using Statistical Analysis System (SAS) of 2012, version 9.4. Data were analysed using One-way Analysis of Variance (ANOVA) in the General Linear Model (GLM) procedure using the following statistical model: $Y_{ij} = \mu + \delta_i + \varepsilon_{ij}$, where Y_{ij} is the dependent variable, μ is the overall mean, δ_i is the effect of diet, and ε_{ij} is the residual error. For all the parameters, significance was set at $P \leq 0.05$ and the Least Square Means (LSMeans) were compared using the probability of difference (PDIFF) option.

Results

Growth performance

Table 2 shows the effect of dietary incorporation of Gly-G-Nano-Ag/TiO₂ on overall FI, BWG, and FCE of broiler chickens fed diets contaminated with aflatoxin B₁. The repeated measurements showed no diet $\times$ week interaction effects on all performance parameters ($P > 0.05$). Hence, the data were presented as overall means across weeks. Similarly, there was no effect of diet ($P > 0.05$) on overall FI, BWG, and FCE.

Carcass Traits and Visceral Organs

The effects of dietary incorporation of Gly-G-Nano-Ag/TiO₂ on carcass traits and visceral organs of broiler chickens fed diets contaminated with aflatoxin B₁ are shown in Table 3. The results showed no effects of dietary treatment ($P > 0.05$) on all the carcass and internal organ parameters measured.

Table 2. Effects of dietary incorporation of glycine-coated green Ag/TiO₂ hybrid nanoparticles on overall feed intake, body weight gain, and feed conversion efficiency of broiler chickens fed diets contaminated with aflatoxin B₁.

Parameter	Dietary treatment			SEM	P-value
	-AF	+AF	+AF+Gly _n		
Initial weight (g/bird)	727.00	736.00	728.00	17.932	0.433
Overall FI (g/bird)	3073.00	3018.50	2959.00	66.693	0.493
Overall BWG (g/bird)	1935.50	1901.00	1845.50	46.710	0.430
Overall FCE	0.63	0.63	0.63	0.011	0.906

AF = aflatoxin B₁; BWG = body weight gain; FCE = feed conversion efficiency; FI = feed intake; Gly_n = glycine-coated green Ag/TiO₂ hybrid nanoparticles; SEM = standard error of the mean.

Table 3. Effects of dietary incorporation of glycine-coated green Ag/TiO₂ hybrid nanoparticles on carcass characteristics (% of CCW, unless stated otherwise) and weights and lengths of internal organs (% of HCW, unless stated otherwise) of broiler chickens fed diets contaminated with aflatoxin B₁.

Parameter	Dietary treatment			SEM	P-value
	-AF	+AF	+AF+Gly _n		
Carcass characteristics					
Slaughter weight (g)	2662.50	2657.50	2576.50	54.602	0.471
Hot carcass weight (g)	1907.50	1868.80	1837.50	49.485	0.612
Cold carcass weight (g)	1897.50	1857.50	1823.80	49.189	0.578
Dressing %	71.62	70.39	71.30	1.251	0.773
Breast weight	22.28	22.42	22.51	0.445	0.933
Wing weight	5.39	5.46	5.35	0.117	0.808
Wing length (cm)	21.21	21.13	20.81	0.204	0.349
Thigh weight	8.14	8.07	7.70	0.196	0.262
Thigh length (cm)	9.58	9.53	10.16	0.401	0.484
Drumstick weight	6.82	6.67	6.70	0.125	0.670
Drumstick length (cm)	11.70	11.54	11.51	0.237	0.830
Back length (cm)	21.03	20.39	20.73	0.451	0.614
Internal organs					
Liver weight	2.61	2.63	2.71	0.085	0.709
Gizzard weight	2.24	2.15	2.12	0.102	0.711
Spleen weight	0.19	0.20	0.19	0.018	0.775
Proventriculus weight	0.58	0.57	0.55	0.031	0.844
Duodenum weight	1.10	1.01	1.07	0.037	0.198
Jejunum weight	2.00	1.81	1.88	0.098	0.399
Ileum weight	1.66	1.43	1.57	0.110	0.331
Caecum weight	0.81	0.80	0.71	0.052	0.365
Colon weight	0.15	0.13	0.13	0.012	0.486
Duodenum length (cm)	30.83	30.85	31.07	0.638	0.957
Jejunum length (cm)	79.62	76.48	78.46	1.588	0.386
Ileum length (cm)	79.88	73.48	77.26	3.186	0.378
Caecum length (cm)	28.10	28.73	28.27	3.397	0.991
Colon length (cm)	5.64	5.11	4.78	0.388	0.309

AF = aflatoxin B₁; Gly_n = glycine-coated green Ag/TiO₂ hybrid nanoparticles; SEM = standard error of the mean.

Haemato-biochemistry parameters

The effects of dietary incorporation of Gly-G-Nano-Ag/TiO₂ on the haematology and serum biochemistry of broiler chickens fed diets contaminated with AFB₁ are presented in Table 4. The results showed that dietary AFB₁

decreased white blood cells and serum albumin, both of which were restored by incorporation of Gly-G-Nano-Ag/TiO₂ ($P < 0.05$ and $P < 0.001$, respectively). Similarly, dietary AFB₁ decreased blood lymphocytes but this parameter was however not restored by Gly-G-Nano-Ag/TiO₂ incorporation ($P < 0.001$). In

contrast, both AFB₁ and Gly-G-Nano-Ag/TiO₂ increased blood heterophils and platelets ($P < 0.001$, respectively). Otherwise, both AFB₁ and the green bimetallic nanoparticles had no

influence on all other haemato-biochemistry variables ($P > 0.05$).

Table 4. Effects of dietary incorporation of glycine-coated green Ag/TiO₂ hybrid nanoparticles on haemato-biochemical parameters of broiler chickens fed diets contaminated with aflatoxin B₁.

Parameter	Dietary treatment			SEM	P-value
	-AF	+AF	+AF+Gly _n		
Haematology					
Haematocrit (%)	29.00	28.25	27.25	0.845	0.358
White blood cells (×10 ⁹ /L)	15.05 ^a	10.98 ^b	12.73 ^{ab}	1.010	P < 0.05
Heterophils (×10 ⁹ /L)	55.13 ^b	76.00 ^a	85.93 ^a	4.356	P < 0.001
Lymphocytes (×10 ⁹ /L)	35.50 ^a	16.75 ^b	8.50 ^b	3.789	P < 0.001
Monocytes (×10 ⁹ /L)	4.94	7.00	5.50	0.949	0.304
Platelets (K/μL)	25.11 ^b	35.09 ^a	31.19 ^{ab}	1.743	P < 0.001
Eosinophils (×10 ⁹ /L)	3.56	0.00	0.50	2.850	0.355
Serum biochemistry					
Albumin (g/L)	14.42 ^a	13.02 ^b	14.40 ^a	0.213	P < 0.001
Alkaline phosphate (U/L)	1942.17	1966.75	1849.92	35.623	0.970
Alanine transaminase (U/L)	15.67	9.33	12.17	3.052	0.364
Amylase (U/L)	530.17	565.83	491.00	69.700	0.754
Lipase (U/L)	10.75	20.42	19.67	5.067	0.350
Blood glucose (mmol/L)	0.47	0.50	1.08	0.234	0.152
Cholesterol (mmol/L)	2.58	2.48	2.37	0.141	0.566
Total protein (g/L)	29.98	29.82	27.52	1.467	0.433
Total bilirubin (mmol/L)	3.58	3.58	2.83	0.369	0.282

^{ab} Means within a row with different superscripts are significantly different ($P < 0.05$); AF = aflatoxin B₁; Gly_n = glycine-coated green Ag/TiO₂ hybrid nanoparticles; SEM = standard error of the mean.

Meat quality

Table 5 shows the effect of including Gly-G-Nano-Ag/TiO₂ in aflatoxin B₁ contaminated diets on broiler meat quality. Data showed that there was no effect of diet ($P > 0.05$) on all meat quality variables of birds at both 45 min and 24 h post-slaughter.

Table 5 Effects of dietary incorporation of glycine-coated green Ag/TiO₂ hybrid nanoparticles on meat quality parameters of broiler chickens fed diets contaminated with aflatoxin B₁.

Parameter	Dietary treatment			SEM	P-value
	-AF	+AF	+AF+Gly _n		
Temperature _{45 min} (°C)	27.80	28.25	28.21	0.472	0.759
pH _{45 min}	5.77	5.81	5.93	0.062	0.193
<i>L</i> * _{45 min}	44.13	44.86	43.26	0.880	0.449
<i>a</i> * _{45 min}	2.75	2.44	2.10	0.368	0.563
<i>b</i> * _{45 min}	2.88	2.89	2.60	0.318	0.774
H _{45 min}	0.85	0.85	0.72	0.088	0.479
C _{45 min} (°)	11.32	12.16	10.21	1.912	0.774
Temperature _{24 h} (°C)	9.33	9.14	9.18	0.24	0.848
pH _{24 h}	6.01	6.06	6.02	0.028	0.517
<i>L</i> * _{24 h}	51.11	51.09	53.65	1.021	0.389
<i>a</i> * _{24 h}	4.16	3.95	5.20	0.530	0.225
<i>b</i> * _{24 h}	4.33	4.14	3.96	0.363	0.779
H _{24 h}	0.84	0.79	0.67	0.065	0.182
C _{24 h} (°)	22.94	23.15	21.48	3.285	0.927
Cooking loss (%)	26.43	26.38	27.36	1.160	0.799
Water holding capacity (%)	82.89	80.71	79.20	2.113	0.476
Drip loss (%)	4.05	5.48	4.94	1.107	0.661
Shear force (N)	9.54	8.28	7.17	0.979	0.254

AF = aflatoxin B₁; Gly_n = glycine-coated green Ag/TiO₂ hybrid nanoparticles; *a** = redness; *b** = yellowness; C = Chroma; H = hue angle; *L** = lightness; SEM = standard error of the mean.

Discussion

This study aimed at investigating the efficacy of Gly-G-Nano-Ag/TiO₂ in mitigating adverse effects of AFB₁ in broiler chickens in terms of productive performance, carcass characteristics, organ development, health-nutrition-related blood physiology, and meat quality. Contrary to previous studies demonstrating detrimental effects of AFB₁ on broiler FI, BWG, FCE, carcass traits, internal organs and meat quality (Liu et al., 2016; Sarker et al., 2021), our results showed no effects of the toxic fungal metabolite on all these variables. However, our findings are not unprecedented. Previously, lack of deleterious effects of dietary feeding of AFB₁ on broiler performance (Magnoli et al., 2011), carcass characteristics and

internal organs (Zhou et al., 2021) as well as meat quality (Fan et al., 2013) has been demonstrated. The dietary concentration of AFB₁ that was tested in the current study was probably too low to elicit significant effects on the measured bird responses. Nonetheless, AFB₁ dosage was within the range of permissible levels (≤ 0.1 mg/kg) for poultry as established by the FDA and European Union regulatory bodies (Gruber-Dorninger et al., 2019; Xie et al., 2022). Otherwise, higher AFB₁ doses (100 to 1500 μ g/kg) have previously elicited adverse effects on productive performance and other attributes of broilers (Fouad et al., 2019). The absence of significant effects of AFB₁ on growth performance, carcass characteristics, visceral organs, and

meat quality despite alterations in selected blood parameters suggests that the birds were subjected to a subclinical rather than a severe aflatoxicosis challenge. The dietary AFB₁ concentration used in the present study (0.04 mg/kg) was considerably lower than levels frequently reported to impair productive performance in broilers and the exposure period was limited to the finisher phase (21 days). Consequently, the challenge may have been sufficient to induce subtle physiological and immunological disturbances, as evidenced by reductions in white blood cells, lymphocytes, and serum albumin, without causing overt production losses. Under such conditions, the ameliorative effects of Gly-G-Nano-Ag/TiO₂ on selected haematological and biochemical indices may indicate protection against early physiological perturbations that precede measurable effects on growth and carcass traits. However, a limitation of the present study is that intestinal histomorphological parameters, were not evaluated. Since AFB₁ can compromise intestinal integrity and nutrient absorption, future studies should therefore incorporate intestinal histomorphometry to better explain the response of broilers to aflatoxicosis and nanoparticle supplementation. Furthermore, the use of a single dietary AFB₁ concentration represents a limitation of the present study; therefore, future investigations should evaluate multiple contamination levels and longer exposure periods, including earlier production phases, to better

characterize dose-dependent responses and the protective efficacy of Gly-G-Nano-Ag/TiO₂.

We further measured the haematological and serum biochemistry parameters as key indicators of the impact of aflatoxicosis in broilers (Yu et al., 2021) and these were the only variables that significantly responded to AFB₁ dosage. The observed decrease in serum albumin, white blood cells, and blood lymphocytes in contrast to increased blood heterophils and platelets concurred with previous findings indicating inhibitory effects of AFB₁ on protein synthesis (Edrington et al., 1997; Kececi et al., 1998; Safameher, 2008; Zhou et al., 2021) and haemopoiesis leading to perturbations in the production of immune cells (Kubena et al., 1990; Kececi et al., 1998; Oguz et al., 2000; Gholami-Ahangaran & Zia-Jahromi, 2014). Indeed, aflatoxicosis is known to induce lymphocytopenia and monocytopenia and to increase heterophil counts through eliciting an inflammatory response not only in chickens but also in ruminants (Basmacioglu et al., 2005). Aside AFB₁-induced perturbations of protein synthesis and haemopoiesis, the decrease in serum albumin, quantitatively the most important extracellular antioxidant (Bourdon and Blache, 2001), might be due to breakdown of the protein upon its binding to and oxidation by the aflatoxin, leading to loss of its antioxidant capacity (Roche et al., 2008). Therefore, our data suggest that apart from inducing perturbations in

protein metabolism and haemopoiesis, dietary AFB₁ would have induced oxidative stress and inflammation in the birds, similarly to previous observations (Li et al., 2019).

Interestingly, our results demonstrated, for the first time, the attenuative effects of dietary Gly-G-Nano-Ag/TiO₂ against deleterious effects of AFB₁ on white blood cells and serum albumin in broilers. This outcome would have been achieved through the nanoparticles employing their high surface area-to-volume ratio to tenaciously adsorb AFB₁ or its AFBO radical (Puzyr et al., 2007; Gibson et al., 2011). This would thus circumvent hepatic metabolism of the aflatoxin and its metabolite leading to their elimination from the body without induction of toxicity to the chickens. Alternatively, the multitudinous phyto-compounds (i.e. reducing sugars, phenolics, carotenoids, alkaloids, triterpenes, hydrogen cyanide, steroids, saponins, oxalate, phytate, tannins, para-pentylphenyl-benzoate, 2-hydroxy-4-methoxybenzaldehyde, (-)-loliolide, 5-chloropropacin, 9-hexacosene, 2-hexen-1-ol, propacin, isovanillin, heptacosane and others) derived from *M. whitei* (Deeh et al., 2024) that were used as Ag/TiO₂ hybrid nanoparticle reducing and stabilizing agents would have served as antioxidants that quenched AFBO-induced oxidative stress. Thirdly, our green bimetallic nanoparticles were coated with glycine, the precursor for GSH (Wu, 2021), which would have detoxified AFBO through conjugation, biodegradation and excretion of the

epoxide through the mercapturic acid pathway (Hayes et al., 1994; Buetler et al., 1995; Manson et al., 1997).

Notwithstanding, the observed inability of Gly-G-Nano-Ag/TiO₂ to ameliorate the detrimental effects of dietary AFB₁ on blood lymphocytes and their increase of blood heterophils and platelets suggests that the green nanohybrids somehow unleashed some oxidative stress and inflammation on the chickens. In this connection, whilst green nanoparticles are widely believed to be non-toxic and safe to use in biological systems, they have often been shown to induce toxicity to cells and organisms (Dmochowska et al., 2020; Machado et al., 2021; Zavitri et al., 2023) through their intracellular dissolution into free ions and formation of ROS that induce oxidative stress (von Hellfeld et al., 2020) and inflammation (Pandey and Mishra, 2022). In our laboratory, green *Mucuna pruriens utilis* seed extract-mediated zinc oxide nanoparticles administered to beef cattle through the feed induced toxicity as demonstrated in decreased bovine growth performance, carcass characteristics, as well as blood MCV, MCH, and neutrophils (Gamedze et al., 2024). Even in plants, green nanoparticles including the nanohybrids have elicited toxic effects as demonstrated in their interference with mitosis in the roots (Singh, 2022). As the nanoparticles used in the current study were a composite of Ag and TiO₂, each of the component nanomaterials would have synergistically contributed to the oxidative stress and inflammation in the birds. Indeed, the enhanced

toxicity of Ag/TiO₂ has been ascribed to the interaction of ROS on the TiO₂ surface and the released Ag⁺ ions, which together multiply the catalytic (toxic) efficiency of the hybrid (Khwanmuang et al., 2017; Prakash et al., 2022). However, we did not measure oxidative stress and inflammatory biomarkers to provide a mechanistic explanation of the observed toxic effects of Gly-G-Nano-Ag/TiO₂. Nonetheless, having observed no deleterious effects on most haemato-biochemistry variables of the birds, as was also observed with all other measurements, we can confidently conclude that our glycine-coated green bimetallic nano-hybrids were generally safe for the broiler chickens, at least at finisher phase.

Conclusion

Dietary administration of AFB₁ and Gly-G-Nano-Ag/TiO₂ induced no effects on growth performance, carcass characteristics, internal organs, meat quality, and most haemato-biochemistry variables but increased blood heterophils and platelets of broiler chickens as the aflatoxin decreased their blood lymphocytes. The green bimetallic nanoparticles however attenuated detrimental effects of AFB₁ on white blood cells and serum albumin.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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