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Evaluation of the efficacy of hydrated sodium calcium aluminosilicate at mitigating the negative impact of aflatoxicosis on nutrient digestibility and other production- and health-related indices in broiler chickens

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Primary Audience: Poultry Producers, Feed Manufacturers, Poultry Veterinarians

SUMMARY

Dietary supplementation with aluminosilicates is a field-practical and cost-effective strategy to reduce the toxicity of feedborne aflatoxins in poultry. Importantly, not all types of aluminosilicates have the same decontaminating efficiency; thus, a full characterization of the protective properties of each single material would assist in selecting the most appropriate binder. Hydrated sodium calcium aluminosilicate (**HSCAS**) has been proven protective against many of the deleterious effects produced by aflatoxin B_1 (**AFB_1**) in broiler chickens. However, to date, there is no information specifically concerning its ability to counteract AFB_1-induced impairment of nutrient digestibility in these animals. Therefore, expanding on previous research, we sought to fill this gap by incorporating new analyses on nutrient digestibility in a typical panel of aflatoxicosis-relevant endpoints. The aflatoxicated chickens that did not receive HSCAS showed many of the commonly reported signs of aflatoxicosis, including growth depression, liver injury, impaired immune function. Interestingly, some less common aflatoxicosis manifestations were also observed, such as increased serum glucose and cholesterol levels, and increased relative weight of abdominal fat. An unexpectedly low

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sensitivity to the challenge with AFB_1 was recorded for the digestibility-related parameters. In AFB_1-exposed chickens that received HSCAS, most of the abovementioned signs of aflatoxicosis were not observed, and the few still-occurring ones were substantially mitigated. Interestingly, some of the production- and health-related variables investigated showed significant improvement even when compared with control chickens. Overall, this study brings new knowledge regarding the potential manifestations of aflatoxicosis in broiler chickens and spectrum of HSCAS' beneficial effects, thereby contributing to better identification and control of an aflatoxin problem in poultry farms.

Key words: poultry, aflatoxin, decontamination, mycotoxin binder, feed additive

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DESCRIPTION OF PROBLEM

Aflatoxins (**AF**) frequently occur as natural contaminants in poultry feeds (Gourama and Bullerman, 1995; Ledoux et al., 1998; Murugesan et al., 2015; Zabiulla et al., 2021) and represent a worldwide threat to the broiler industry (Bryden, 2012). More specifically, AF are a class of major mycotoxins primarily produced by fungal species of the genus *Aspergillus* (*A. flavus* and *A. parasiticus*). There are many types of naturally occurring AF, but the 4 main ones are aflatoxin B_1 (**AFB_1**), aflatoxin B_2 (**AFB_2**), aflatoxin G_1 (**AFG_1**), and aflatoxin G_2 (**AFG_2**), with AFB_1 being the most prevalent and the most toxic (Ledoux et al., 1998; Phillips et al., 2002; Abrar et al., 2013; Logrieco et al., 2018; Xu et al., 2022).

Ingestion of AFB_1-contaminated feeds by young broiler chickens often results in decreased feed intake and conversion efficiency, growth retardation, poor carcass characteristics, and increased morbidity and mortality rates, eventually leading to considerable economic losses (Bryden, 2012; Fouad et al., 2019; Liu et al., 2020; Zabiulla et al., 2021; Alharthi et al., 2022). Moreover, the carry-over of feed-borne AFB_1 and its metabolites to animal-derived edible products poses an important food safety issue, especially considering the mutagenic and carcinogenic potential of AFB_1 (Phillips et al., 2002; Pimpukdee et al., 2004; Zabiulla et al., 2021; Alharthi et al., 2022; Sarker et al., 2023).

The negative impact of aflatoxicosis on commercial broiler production is the likely expression of the capacity of AFB_1 to compromise various fundamental aspects of poultry health through multiple actions that are of both genotoxic and nongenotoxic nature (DNA damage, inhibition of RNA and protein synthesis, free radical overproduction with consequent oxidative damage) (Phillips et al., 2002; Verma et al., 2002; Rashidi et al., 2020; Hassan et al., 2021; Zabiulla et al., 2021; Alharthi et al., 2022). Liver, kidneys, and lymphoid organs are well-known targets of AFB_1 toxicity (Fouad et al., 2019; Zabiulla et al., 2021). Indeed, aflatoxicosis in poultry species is typically associated with hematochemical alterations and pathological lesions that are indicative of hepato-renal damage, as well as with increased susceptibility to infectious diseases, which reflects immune system suppression (Ledoux et al., 1998; Rahim et al., 1999; Liu et al., 2020; Zabiulla et al., 2021; Alharthi et al., 2022). In addition, there is substantial evidence that a contribution to the detrimental influence of aflatoxin exposure on animal health and growth performance results from AFB_1-induced damage to the intestine (Applegate et al., 2009; Celi et al., 2017; Alharthi et al., 2022; Ducatelle et al., 2023; Sarker et al., 2023). Loss of intestinal architectural and functional integrity causes, among others, disruption of nutrient digestion and/or absorption processes (Huff et al., 1992; Liu et al., 2018; Alharthi et al., 2022; Xu et al., 2022; Sarker et al., 2023) and this, in turn, affects the sustainability of the whole production system by also increasing feed costs and environmental impact of undigested dietary proteins (Vieira et al., 2023).

The inclusion of aluminosilicate minerals as feed additives into poultry diets is presently regarded as one of the most field-practical and cost-effective strategies to reduce the toxicity of

the feed-contaminating AF (Pimpukdee et al., 2004; Gilani et al., 2016; Phillips et al., 2019; Zabiulla et al., 2021; Xu et al., 2022). These inorganic, non-nutritive, and inert materials function as aflatoxin adsorbing agents, decontaminating the feed by directly binding the AF that may be present through ion exchange. The mycotoxin molecules that have thus been trapped and neutralized into insoluble toxin-binder complexes are excreted harmlessly via the fecal route, while the overall amount of free biologically active mycotoxin molecules that are available in the gastrointestinal tract for action, interactions, and absorption is considerably reduced (Alharthi et al., 2022; Xu et al., 2022).

Aluminosilicate-based adsorbents encompass a wide variety of structurally and chemically diverse types of clay minerals, including bentonite, zeolite, smectite, and hydrated sodium calcium aluminosilicate (**HSCAS**) (Scheideler, 1993; Phillips et al., 2002; Gilani et al., 2016; Xu et al., 2022; Kihal et al., 2022). Importantly, not all types of aluminosilicates are the same in terms of decontaminating potential (Ledoux et al., 1998; Zabiulla et al., 2021); therefore, a full characterization of the protective properties of each single material would assist in the selection of the most appropriate binder during the management of an aflatoxin problem in practical field situations (Scheideler, 1993; Gilani et al., 2016).

HSCAS, originally used as a feed anticaking additive (Kubena et al., 1990; Pimpukdee et al., 2004), is one of the aluminosilicates that has received the most attention as an aflatoxin binder (Chen et al., 2014) due to a set of desirable characteristics (Scheideler, 1993; Miles and Henry, 2007; Gilani et al., 2016; Phillips et al., 2019), including: (a) a high binding affinity and capacity for AFB_1; (b) the stability of the complexes formed with AFB_1; (c) the GRAS (generally recognized as safe) status; (d) the little to no interference with intestinal absorption of essential dietary micronutrients (such as vitamins, amino acids and minerals) at dietary inclusion levels up to 1%. The efficacy of HSCAS at protecting broiler chickens against the toxicity of AFB_1 has been demonstrated in several studies for many of the deleterious effects that this mycotoxin is known to produce in poultry (Scheideler, 1993; Rahim et al., 1999; Chen et al., 2014; Gilani et al., 2016; Phillips et al., 2019; Hassan et al., 2021). However, to date, there is no information in the published literature specifically concerning the protective efficacy of this type of adsorbent against AFB_1-induced impairment of nutrient digestibility in broiler chickens. By contrast, this specific aspect has recently been explored for other aluminosilicate-based mycotoxin binders, namely bentonite, zeolite, and smectite (Liu et al., 2018; Alharthi et al., 2022).

Based on the above considerations, the present study was carried out to test HSCAS for its ability to mitigate the negative impact of AFB_1 on apparent total tract digestibility of nutrients in broiler chickens, by incorporating new analyses on nutrient digestibility in a typical panel of aflatoxicosis-relevant endpoints (various productivity indices, serum markers of immune and hepato-renal health, histomorphology of some lymphoid organs and liver).

MATERIALS AND METHODS

Animals and Experimental Design

A total of six hundred one-day-old male broiler chicks (Ross 308) were randomly assigned to one of the following 3 dietary treatment groups: G1 [control chicks, fed basal commercial feed formulated according to the strain requirements (Table 1)]; G2 [aflatoxicated chicks, fed basal feed experimentally contaminated with AFB_1]; G3 [supplemented chicks, receiving the same AFB_1-contaminated feed as G2, but supplemented with the aflatoxin binder HSCAS at the dietary inclusion level of 2 g/kg feed (0.2%) as recommended by its manufacturer (Rota Medencilik Tarim Hay VAN CILIK, Turkey)].

Each experimental group consisted of 200 birds, equally divided into 8 separate replicate pens (25 birds each, with a stocking density of 10 birds/m^2). The birds of each replicate were housed under suitable environmental conditions (23 h light−1 h dark cycle in an open-door building; 24−26°C daily temperature and 60 −70 % humidity), for a total of 35 d (5 wk) of feeding (unless otherwise stated), with feed and water being provided *ad libitum*.

Table 1. Composition and chemical analysis of the basal commercial feed.

Ingredient (%)	Starter 0−14 d	Grower 15−24 d	Finisher 25−33 d
Yellow corn	53.500	58.551	61.000
Soybean meal, 46%	38.951	34.270	31.300
Corn gluten meal, 60%	1.760	1.960	1.550
Soya oil	1.004	1.797	2.980
Calcium carbonate	1.463	1.323	1.230
Mono calcium phosphate	1.030	0.840	0.716
Salt	0.262	0.290	0.296
Sodium bicarbonate	0.130	0.094	0.090
DL Methionine, 99%	0.299	0.262	0.237
L-Lysine HCl, 98%	0.155	0.175	0.164
L-Threonine	0.061	0.053	0.052
Premix[1]	0.3	0.3	0.3
Anticoccidia (diclazuril)	0.07	0.07	0.07
Phytase 500 FTU	0.015	0.015	0.015
Total	100	100	100
Chemical analysis on DM basis:			
AME kcal	2915	3020	3120
Crude protein, %	23.02	21.11	19.14
Fat, %	3.883	4.761	5.95
Digestible LYS, %	1.4	1.29	1.2
Digestible Methionine and Cysteine, %	1.008	929	0.864
Digestible THR, %	0.952	0.929	0.864
Digestible ARG, %	1.486	1.354	1.26
Digestible ILE, %	0.997	0.91	0.842
Digestible LEU, %	1.849	1.734	1.607
Digestible VAL, %	1.106	1.019	0.948
Calcium, %	0.96	870	0.81
Available P, %	0.48	0.435	0.405
Sodium, %	0.16	0.16	0.16
Chloride, %	0.25	0.25	0.23

[1]Multiveta (Multiveta, Cairo, Egypt). Composition (per 2 kg): Vitamin A 12,000,000 IU; vitamin D3 2,500,000 IU; vitamin E 10,000 mg; vitamin K3 2000 mg; vitamin B1 1000 mg; vitamin B2 5000 mg; vitamin B6 1500 mg; vitamin B12 10 mg; niacin 30,000 mg; biotin 50 mg; folic acid 1000 mg; pantothenic acid 10,000 mg; manganese 60,000 mg; zinc 50,000 mg; iron 30,000 mg; copper 4000 mg; iodine 300 mg; selenium 100 mg; cobalt 100 mg.

All chickens were subjected to the following vaccination program: (a) hatchery vaccination with Bursaplex® (Zoetis, Taguig City, Philippines), for prevention of infectious bursal disease (**IBD**), and Newflend® ND H9 (Boehringer Ingelheim India Pvt. Ltd., Mumbai, India), for prevention of Newcastle Disease (**ND**) and avian influenza (**AI**); (b) 1-day-old chick vaccination with NOBILIS® IB 4−91 and MA5 + CLONE 30 (MSD Animal Health Phils. Inc., Makati, Philippines), for prevention of avian infectious bronchitis (including that caused by serotype Massachusetts, strain Ma5), and ND; (c) at 10 d of age, vaccination with cloned live NOBILIS® ND LaSota (MSD Animal Health Phils. Inc., Makati, Philippines), for prevention of ND (Strain Clone 30); (d) at 14 d of age, vaccination with Bursine Plus® (Zoetis, Taguig City, Philippines), for prevention of IBD.

The animal study was conducted following the National Institutes of Health (**NIH**) animal care and handling guidelines and was approved by the Institutional Animal Care and Use Committee of the University of Sadat City, Egypt (Approval No. 4/2016EC).

Preparation of AFB_1-Contaminated Feed

AFB_1 was produced by liquid culture of a standard toxigenic strain of *A. flavus* as reported previously (Ismail et al., 2020). Immunoaffinity columns (Aflatest) combined with an HPLC apparatus were used for its extraction and purification from the culture medium and quantification of the amount produced (Ekwomadu et al., 2021). Pure analytical standards of AF (AFB_1, AFB_2, AFG_1, and AFG_2) were purchased from Sigma Chemical Company.

Fifty milligrams of AFB_1 were dissolved in 50 mL of benzene, and this solution was added to 250 g of basal feed. After completely evaporating the solvent under an exhaust fan overnight, this material was added to 20 kg of the ration to obtain a final AFB_1 concentration of 2.5 ppm. A micro-mixer was used to homogenize the feed and the toxin to ensure even distribution at the desired ratio (Ismail et al., 2020). Before use in the feeding trial, the basal diet was analyzed for possible basal contaminating levels of AFB_1 and other mycotoxins using a validated multianalyte method (Gruber-Dorninger et al., 2023). AFB_1 was the only type of mycotoxin detected, and it was found to be present at a level of 1.0 ppb. All other mycotoxins, including ochratoxins, T-2 toxin, and fumonisins, were below the limits of detection.

Growth Performance Indices and Carcass Characteristics

Body weight (**BW**) was recorded at the beginning of the trial and then at the end of the

whole experimental period (5 wk in total) to calculate the overall BW gain (**BWG**) and the average daily weight gain (**ADWG**). In parallel, feed consumption was also recorded to calculate average daily feed intake (**FI**) and feed conversion ratio (**FCR**). At the end of the feeding trial (35 d), 16 chickens/treatment (2 chickens/replicate pen, selected among those with BW close to the average BW of the pen) were slaughtered and dissected to measure the weights of the whole carcass, as well as of some carcass components, including breast muscles, abdominal fat, liver and immune organs (spleen, bursa of Fabricius and thymus). All carcass-related weights were expressed as a percentage of the pre-slaughter BW (relative weights).

Nutrient Digestibility

At 32 d after the beginning of the feeding trial, 2 chicks/replicate pen (16 chicks/group) were randomly selected, weighed individually, and transferred to special batteries containing individual cages (metabolic cages) for the digestibility experiment, during which the experimentally assigned type of feed and water continued to be offered *ad libitum*. After a 24-h acclimatization, excreta were collected from each cage for 3 consecutive days. Feed consumption was recorded during the whole collection period. The total amount of excreta collected per group was mixed and oven-dried for subsequent chemical analyses.

Both dried excreta and experimental diets were analyzed for dry matter (**DM**), crude protein (**CP**), and crude fiber (**CF**), with the latter 2 parameters expressed on a DM basis to correct for differences in moisture content. The proximate analysis of CP and CF was conducted using AOAC official methods 976.05 and 962.09, respectively (AOAC, 2000a, b).

To calculate the apparent digestibility of each specific nutritional component considered [namely, dry matter digestibility (**DMD**), protein retention (**PR**), and crude fiber utilization (**CFU**)], the analytical values were plugged into the following general equation: nutrient digestibility (%) = (total nutrient intake − total nutrient excreted)/total nutrient intake × 100.

Serum Markers of Immune and Hepato-Renal Health

At weekly intervals starting from the first week of the feeding trial (namely at 7, 14, 21, 28, and 35 d of chicks' age), blood samples were taken by syringes from the wing vein of 40 birds of each experimental group (5 randomly selected birds/replicate pen). Blood was left to coagulate at 37°C for 2 h; after that, it was centrifuged (2,000 x *g*, 10 min, 4°C; MSE Harrier 18/80R) and the resulting serum was kept at −20°C until subsequent analysis. In the so collected serum samples, antibody (**Ab**) titers against ND virus and AI type A viruses (subtypes H5N1 and H9N1) were measured by hemagglutination inhibition test (Loeb et al., 2020).

In addition, serum samples collected on the last day of the feeding trial (d 35; shortly before slaughtering) were also analyzed for: (a) the levels of total cholesterol (Jaďuttová et al., 2019); (b) the activities of alanine transaminase (**ALT**) and aspartate aminotransferase (**AST**) enzymes (Lin et al., 2022); (c) glucose levels (Dev et al., 2020); (d) total protein (**TP**) levels (Abudabos et al., 2018); (e) uric acid levels (Nwaigwe et al., 2020). All parameters were measured colorimetrically using commercial kits (Diamond Diagnostics, Egypt).

Histomorphology of Lymphoid Organs and Liver

From the thymus, bursa, and liver of each chicken slaughtered on d 35 for determination of carcass characteristics (16 chickens/treatment, 2 chickens/replicate pen), small tissue samples were immediately collected for histomorphological examination by light microscopy. Thymus and bursa samples were fixed in Bouin's solution for 18 to 24 h, whereas liver samples (1 × 1 × 1 cm) were fixed in 10% neutral buffered formalin for 48 h. Afterward, fixed samples were dehydrated in ascending alcohol percentage (30, 50, 70, and 90%, and absolute alcohol), cleared in methyl benzoate, and embedded in paraffin wax. Histomorphological examinations were performed on 5 to 7 μm thick sections stained with Harris hematoxylin and eosin. The photomicrographs were acquired

using a Leica digital camera connected to a binocular light microscope.

Statistical Analysis

After testing for normality, all data sets were expressed as means ± SEM. Significance of the differences among groups was evaluated by an ANOVA using the GLM procedure (GraphPad Prism, v. 8; GraphPad Software, Inc., La Jolla, CA). Tukey's multiple comparison test was used to identify which means were significantly different from each other. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Response of Broiler Chickens to AFB$_1$

As shown in Table 2, chickens fed the diet contaminated with 2.5 ppm of AFB_1 (G2) had significantly ($P < 0.05$) reduced final BW, BWG, and ADWG (by 10% on average) compared with those fed the control diet (G1). These changes were also accompanied by a significant ($P < 0.05$) increase in FCR (of about 9.2%), whereas FI remained unchanged.

In an earlier report (Scheideler, 1993), the same level of dietary exposure to AFB_1 (2.5 ppm) also was found to adversely affect chick BW and FCR, however to a greater extent than in our study (+15% and +27%, respectively). Among the differences in the experimental conditions of the 2 studies that may account for the different magnitude of the growth-depressing effects of AFB_1, the different duration of the 2 feeding trials deserves special consideration. Indeed, in the study by Scheideler (1993), as in many other published studies, chickens were exposed to dietary AFB_1 until 3 wk of age, whereas in the present trial the exposure was protracted until birds achieved the commercial age of 5 wk (35 d), and this longer duration probably allowed partial spontaneous recovery. Although some authors reported that adaptive responses to prolonged AFB_1 intake would not occur in chickens (Chen et al., 2014), it is a well-known fact that the responses of broilers to aflatoxin in different literature sources can be inconsistent (Rashidi et al., 2020; Dersjant-Li et al., 2003). Therefore, the proposed explanation remains a possibility.

In both our study and the study by Scheideler (1993), dietary exposure to 2.5 ppm of AFB_1 was found not to affect FI. Conversely, and in corroboration of the inconsistency mentioned above, other studies using comparable levels of AFB_1 exposure (2 mg/kg feed = 2 ppm) reported significant decreases in the amount of feed consumed by AFB_1-exposed chickens (Zhao et al., 2010; Chen et al., 2014). In this case, based on information provided by Rashidi et al. (2020), inter-study differences in diet composition may have played a major role in determining this discrepancy.

Table 2. Effects of hydrated sodium calcium aluminosilicate (HSCAS) on the growth performance of broiler chickens experimentally challenged with aflatoxin B_1 (AFB_1) for 35 d.

	Dietary treatments			
Variables	Control	AFB_1	AFB_1 + HSCAS	*P*-value
Initial BW[1] (g)	39.8 ± 0.5	40.0 ± 0.2	40.1 ± 0.4	0.674
Final BW at 35 d (g)	2,095 ± 87^b	1,885 ± 34^c	2,225 ± 69^a	0.015
BWG[2] at 35 d (g)	2,055.2 ± 87^b	1,845 ± 34^c	2,184.9 ± 68^a	0.018
ADWG[3] (g/d)	58.72 ± 0.92^b	52.71 ± 0.36^c	62.43 ± 0.87^a	0.021
FI[4] at 35 d (g)	4,095 ± 205	4,010 ± 202	4,020 ± 96	0.428
FCR[5] at 35 d	1.95 ± 0.3^b	2.13 ± 0.4^a	1.80 ± 0.2^c	0.032

Data are means (± SEM) for all chickens (200) per group.

Within the same row, significant differences at $*P < 0.05$ are indicated by different superscript letters (a, b, c), whereas non-significant differences are indicated by the same superscript letters or without any letters.

[1]BW: body weight.

[2]BWG: body weight gain.

[3]ADWG: average daily weight gain.

[4]FI: feed intake.

[5]FCR: feed conversion ratio.

Table 3. Effects of hydrated sodium calcium aluminosilicate (HSCAS) on the digestibility of selected nutrients in broiler chickens experimentally challenged with aflatoxin B_1 (AFB_1) for 35 d.

	Dietary Treatments			
Variables	Control	AFB_1	AFB_1 + HSCAS	*P*-value
Dry matter digestibility (%)	63.2 ± 7^{ab}	61.7 ± 5^{b}	69.1 ± 6^{a}	0.048
Protein retention (%)	59.5 ± 9^{ab}	52.2 ± 7^{b}	64.4 ± 7^{a}	0.043
Crude fiber utilization (%)	26.8 ± 4	28.9 ± 4	29.3 ± 6	0.165

Data are means (± SEM) for 16 chickens per group (2 chickens from each replicate).
Within the same row, significant differences at $*P < 0.05$ are indicated by different superscript letters (a, b, c), whereas non-significant differences are indicated by the same superscript letters or without any letters.

The finding that FI was not reduced in our aflatoxicated chickens suggests that potential causes for these animals' reduced growth (and increased FCR) probably lie elsewhere. In this respect, one may think that growth depression could depend on the documented ability of AFB_1 to impair the chickens' digestion and absorption of nutrients from feed (Liu et al., 2018; Alharthi et al., 2022). However, for the aflatoxicated chickens of the present study, this hypothesis seems unlikely. Indeed, our experiments showed that the nutrient digestibility parameters investigated were not (CFU), or only slightly and nonsignificantly (DMD and PR) affected by the exposure to AFB_1 (G2) relative to the control condition (G1) (Table 3). This disagreement between our results and the various studies reporting the occurrence of decreased nutrient digestibility in association with aflatoxicosis in broilers and other poultry species (Verma et al., 2002; Liu et al., 2018; Fouad et al. 2019; Alharthi et al., 2022; Xu et al., 2022) may be explained, once again, in light of inter-study differences in one or more of the numerous interrelated factors that have been reported to influence the animal response to AF (including diet composition, source and dose of AFB_1, chicken strain, age and duration of AFB_1-exposure, presence or absence of other feed-contaminating mycotoxins, and so on) (Verma et al., 2002; Chen et al., 2014; Fouad et al., 2019; Rashidi et al., 2020; Amaro et al., 2023). The fact that intestinal histomorphology was not evaluated in our study must be acknowledged as a limitation, for it would have helped assess the actual extent of the impact of the feed-contaminating AFB_1 on the intestinal integrity of the intoxicated broiler chickens.

Having excluded the hypothesis that the growth-impairing effects of AFB_1 on the chickens of the present study could depend on a decreased nutrient bioavailability, the most plausible explanation for this finding appears to be a compromised nutrient utilization. In this regard, it is known that lipids, carbohydrates, amino acids, and proteins serve as fuel molecules and building blocks for animal growth and development, and that organs such as liver play a major role in maintaining their homeostasis (Majeed et al., 2017; Fouad et al., 2019; Xu et al., 2022). Confirming the well-documented hepatotoxicity of AFB_1 (Fouad et al., 2019; Rashidi et al., 2020), our study revealed hepatocellular damage in the broiler chickens fed the AFB_1-contaminated diet. This, in the first place, was indicated by the microscopic examination of the liver histology. Indeed, while the liver of the broiler chickens receiving the control diet (G1) showed the typical features of normal avian histology (Figure 1a), the liver of the aflatoxicated chickens (G2) exhibited apparent alterations (Figure 1b). More specifically, the hepatocytes showed varying degrees of degeneration, characterized by cytoplasmic vacuolation, swelling, and loss of cellular boundaries. Extensive areas of hepatocellular necrosis were present, characterized by pyknotic nuclei, disrupted cellular architecture, and infiltration of inflammatory cells. Moreover, signs of fibrosis (collagen deposition and fibrous tissue proliferation), inflammatory cell infiltration (predominantly of lymphocytes and heterophils), and increased proliferation of bile ducts were observed. Similar histopathological changes in the liver of AFB_1-exposed chicken were reported by other

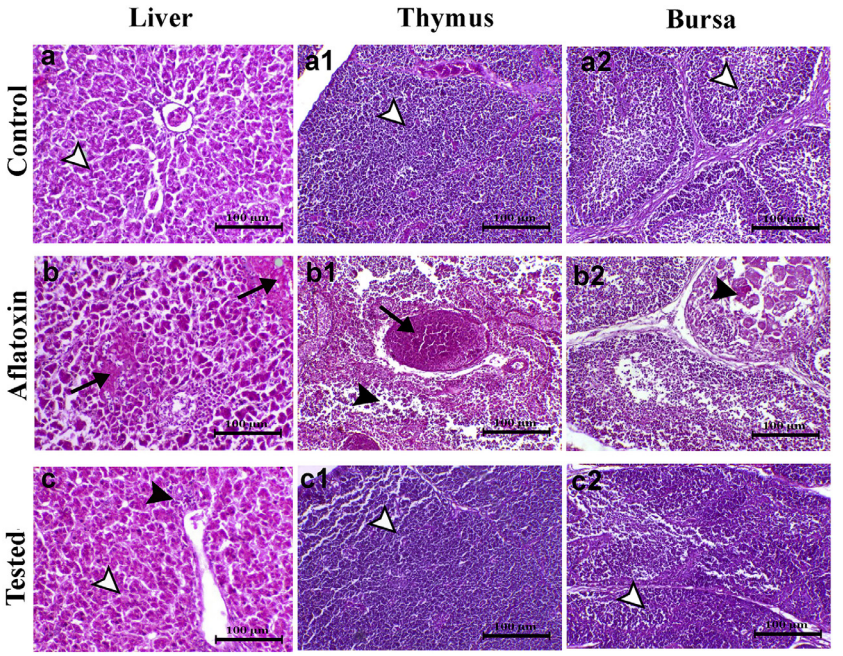

Figure 1. Representative photomicrographs of hematoxylin-eosin stained sections of liver, thymus, and bursa sampled from broiler chickens receiving one of the following experimental dietary treatments for 35 d: control diet (a, a1, a2; white arrowheads indicate normal hepatocytes, normal lymphoid cells within the thymus and bursa), diet experimentally contaminated with aflatoxin B_1 [b, b1, b2; arrows indicate necrosis within the hepatic parenchyma, thymus showing severe congestion and haemorrhage (arrow) and necrosis (black arrowheads) and bursa showing severe necrosis of the lymphoid follicles (black arrowheads], diet experimentally contaminated with aflatoxin B_1 and supplemented with the tested anti-mycotoxin feed additive (hydrated sodium calcium aluminosilicate) (c, c1, c2; black arrowhead indicates limited focal necrosis, white arrowheads within the different image indicate normal parenchyma).

authors (Ledoux et al., 1998; Zhao et al., 2010; Rashidi et al., 2020; Zabiulla et al., 2021).

Another finding of our study that revelaed the occurrence of hepatocellular damage in the aflatoxicated chickens (G2) was the measurement of increased serum activity of the hepatic enzyme ALT compared with that measured in broilers fed control diet (G1) ($P < 0.05$) (Table 4). Serum AST activity, instead, was unaffected by the experimental challenge with AFB_1. Many other studies in the published literature documented increased serum ALT activity in broiler chickens exposed to AFB_1 (Liu et al., 2018; Rashidi et al., 2020; Hassan et al., 2021; Zabiulla et al., 2021; Alharthi et al., 2022). This, however, in most cases was reported to occur in association with parallel increase in serum AST activity. At any rate, one study can be cited (Zhao et al. 2010) that, similar to our study, reported the lack of any changes in serum AST activity following exposure to 2 mg/kg (= 2 ppm) of AFB_1, despite the presence of a severe liver histopathology.

Other serum biochemical alterations that were detected in the aflatoxicated chickens of our study (G2) included higher serum levels of total cholesterol and glucose than in chickens fed control diet (G1) ($P < 0.05$) (Table 4). These findings may suggest that the loss of structural integrity of the liver caused by AFB_1 had actually resulted in derangement of the organ's metabolic functions, particularly of its role in the regulation of lipid and carbohydrate metabolism (Maurice et al., 1983; Majeed et al., 2017; Rashidi et al., 2020; Zabiulla et al., 2021; Alharthi et al., 2022). However, it should be noted that, although reports of increased cholesterol or glucose levels in association with aflatoxicosis can be found in the published literature (Liu et al., 2018 and Maurice et al., 1983,

Table 4. Effects of hydrated sodium calcium aluminosilicate (HSCAS) on selected hematochemical parameters of broiler chickens experimentally challenged with aflatoxin B_1 (AFB_1) for 35 d.

	Dietary Treatments			
Variables	Control	AFB_1	AFB_1 + HSCAS	*P*-value
ALT[1]	235 ± 14^{b}	283 ± 13^{a}	182 ± 12^{c}	0.032
AST[2]	249 ± 14^{a}	248 ± 16^{a}	205 ± 15^{b}	0.016
Total proteins	3.53 ± 0.16^{ab}	3.03 ± 0.18^{b}	3.83 ± 0.12^{a}	0.043
Total cholesterol	139 ± 4^{b}	152 ± 3^{a}	125 ± 5^{c}	0.025
Glucose	145 ± 5.6^{c}	175 ± 3.9^{a}	158 ± 3.1^{b}	0.0439
Uric acid	7.1 ± 0.2^{b}	8.5 ± 0.2^{a}	7.1 ± 0.1^{b}	0.039

Data (mg/dL) are means (± SEM) for 40 chickens per group (5 chickens from each replicate).

Within the same row, significant differences at $^{*}P < 0.05$ are indicated by different superscript letters (a, b, and c), whereas nonsignificant differences are indicated by the same superscript letters or without letters.

[1]ALT: Alanine transaminase.

[2]AST: Aspartate aminotransferase.

respectively), reports of decreased serum levels of cholesterol or glucose in aflatoxicated chickens seem more common (Kubena et al., 1998; Oğuz et al., 2000; Zhao et al., 2010; Chen et al., 2014; Alharthi et al., 2022). Based on the knowledge available from human medicine (Longo et al., 2001), the possibility exists that the hypercholesterolemia observed in the aflatoxicated chickens of our study was not (or not only) due to altered cholesterol metabolism, but rather (or also) to a chronic cholestatic liver disease. This hypothesis seems supported by the finding of histological signs of cholestasis (e.g. bile duct hyperplasia) in the liver of these animals (Figure 1b), that other investigators also reported (Ledoux et al., 1998; Rashidi et al., 2020; Zabiulla et al., 2021). As for the finding of elevated serum glucose levels, the possibility should be considered that a causative contribution to this alteration may derive from a damage that AFB_1 would concomitantly induce to the endocrine portion of the pancreas (Majeed et al., 2017). Including a histomorphological evaluation of this organ in our study would have helped verify this hypothesis. However, there is evidence in the literature that aflatoxicosis in broilers is associated with focal pancreatitis, with compromised integrity of both acinar and islet cells (Fouad et al., 2019; Zabiulla et al., 2021). Moreover, in humans, it has been demonstrated that diabetes is a common sequela of pancreatitis, and that individuals with post-pancreatitis diabetes are characterized by a significantly larger amount of abdominal fat compared with healthy controls (Ko et al., 2021).

Actually, this combination of alterations was found to occur in our study, as the aflatoxicated chickens (G2), in addition to elevated blood glucose levels, also had greater relative abdominal fat weight (increased by about 43%) than control chicks (G1) ($P < 0.05$) (Table 5). Conversely, the relative weights of carcass, breast muscle and internal non-immune organs (liver) were unaffected by the consumption of AFB_1-contaminated diet (Table 5). In the study conducted by Rashidi et al. (2020), the response of chickens to AFB_1 showed a similar pattern, even though with some differences. More specifically, and in line with our study, Rashidi et al. (2020) reported that the AFB_1-induced decrease in live BW was associated with increased relative weight of abdominal fat; however, no significant changes in blood glucose (or cholesterol) concentrations were detected in their study. Moreover, and again consistent with our study, Rashidi et al. (2020) found no changes in the proportional contribution of the whole carcass and liver weights to the whole BW; however, differently from our study, these authors found that the relative weight of breast muscle was reduced. On the other hand, the relative weight of thigh muscle did not change in the study by Rashidi et al. (2020), therefore the possibility cannot be ruled out that just the opposite occurred in the carcass composition of the aflatoxicated birds of our study (i.e. reduced relative thigh muscle weight,

Table 5. Effects of hydrated sodium calcium aluminosilicate (HSCAS) on the relative weights of carcass and its selected components in broiler chickens experimentally challenged with aflatoxin B_1 (AFB_1) for 35 d.

	Dietary treatments			
Variables	Control	AFB_1	AFB_1 + HSCAS	*P*-value
Pre-slaughter BW[1] (g)	1,996 ± 78	1,888 ±42	2,108 ± 68	0.065
Carcass[2]	64.8 ± 1.51	63.2 ± 3.02	64.4 ± 1.1	0.568
Breast muscle[2]	24.0 ± 1.8	23.4 ± 2.2	23.9 ± 2.1	0.432
Abdominal fat[2]	1.38 ± 0.32^{c}	1.98 ± 0.17^{a}	1.71 ± 0.20^{b}	0.043
Liver[2]	1.84 ± 0.11	2.08 ± 0.07	2.10 ± 0.15	0.231
Bursa[2]	0.181 ± 0.01^{ab}	0.158 ± 0.02^{b}	0.252 ± 0.02^{a}	0.047
Thymus[2]	0.5 ± 0.01^{ab}	0.39 ± 0.04^{b}	0.58 ± 0.01^{a}	0.043
Spleen[2]	0.09 ± 0.001^{ab}	0.08 ± 0.001^{b}	0.102 ± 0.03^{a}	0.044

Data are means (± SEM) for 16 chickens per group (2 chickens from each replicate).

Within the same row, significant differences at $*P < 0.05$ are indicated by different superscript letters (a, b, c), whereas non-significant differences are indicated by the same superscript letters or without any letters.

[1]BW: body weight.

[2]% of BW (g/100 g pre-slaughter BW).

in combination with the already mentioned unchanged relative breast muscle weight). At any rate, it must be mentioned that in many of the studies cited so far, differently from ours, and partly also from that of Rashid et al. (2020), the changes induced by aflatoxicosis in the carcass characteristics of broiler chickens did not involve the relative weight of abdominal fat (Zabiulla et al., 2021; Alharthi et al., 2022), but more frequently consisted of increased relative liver weight (Maurice et al., 1983; Kubena et al., 1998; Ledoux et al., 1998; Zhao et al., 2010; Chen et al., 2014; Zabiulla et al., 2021; Alharthi et al., 2022), in association with lowered carcass or breast meat yields (Zabiulla et al., 2021; Alharthi et al., 2022). In this case, too, differences in the experimental conditions between the present study and studies by other authors (e.g. source of aflatoxin, levels of animal exposure to the mycotoxin, duration of the feeding trial, basal diet composition) may account for these conflicting findings regarding the effects of AFB_1 exposure on the overall body and carcass composition of broiler chickens.

A further difference with most of the published literature is that, in our study, aflatoxicosis was associated with only a slight, and non-significant decrease in serum TP levels (Table 4), whereas significantly decreased TP levels are commonly reported in aflatoxicated broiler chickens by other authors (Kubena et al., 1998; Oğuz et al., 2000; Zhao et al., 2010; Chen et al., 2014; Liu et al., 2018; Rashidi et al., 2020; Alharthi et al., 2022). This suggests that, under the experimental conditions adopted in the present study, the AFB_1-induced liver damage did not result in an appreciable impairment of hepatic control of protein synthesis. We were able to find only one study in the literature (Zabiulla et al., 2021) which reported similar results, however using a much lower level of AFB_1 exposure (0.5 ppm).

Despite the lack of changes in the serum TP levels, the serum levels of uric acid resulted to be significantly increased in the aflatoxicated chickens of our study (G2) compared with levels measured in control chicks (G1) ($P < 0.05$) (Table 4), which is consistent with some previous reports (Rashidi et al., 2020; Hassan et al., 2021). Uric acid is a product of protein catabolism, but just in light of the TP-related results, it seems unikely that the increased levels of this catabolite could reflect a disturbed protein and amino acid metabolism consequent to AFB_1-induced liver damage (Charlton, 1996; Singh et al., 2019). A more likely explanation for this finding of ours may lie in concomitant aflatoxin-induced kidney damage, with consequent impairment of renal excretory functions (Singh et al., 2019; Hassan et al., 2021). Indeed, kidneys have a key role in the excretion of uric acid (Singh et al., 2019), and the nephrotoxicity of AFB_1 in broiler chickens is well-documented (Kubena et al., 1998; Ledoux et al., 1998; Liu et al., 2018; Fouad et al., 2019; Zabiulla et al.,

2021). A histomorphological examination of kidney tissue and/or the measurement of other serum biochemical markers of renal functionality (e.g. creatinine and urea nitrogen) would have helped verify this hypothesis, but were not planned for this study.

The findings of the present study, instead, provided enough evidence to confirm the well-known immunotoxicity of AFB_1 for broiler chickens (Fouad et al., 2019; Kraft et al., 2021; Sun et al., 2022). Indeed, the microscopic examination of the selected lymphoid organs (thymus and bursa of Fabricius) revealed the presence of alterations indicative of tissue damage, that were similar to those reported by other authors (Zabiulla et al., 2021). More specifically, as opposed to the normal histological appearance of the thymus and bursa collected from the chickens fed the control diet (G1) (Figure 1a1 and 1a2), the thymus gland of the aflatoxicated chickens (G2) showed marked lymphocyte depletion in the cortex, with decreased cellularity and disruption of the normal architecture (Figure 1b1). Additionally, an increase in the number of apoptotic cells was observed. Similarly, the microscopic examination of the bursa (Figure 1b2) revealed a decreased number and size of follicles, suggesting follicle atrophy, along with significant decrease in lymphocytes within the follicles, indicating lymphoid depletion. Moreover, the bursal follicles appeared shrunken and distorted, with widened interfollicular spaces due to edema. Epithelial architecture disruption was also evident, with the bursal epithelium exhibiting loss of integrity and degenerative changes, including vacuolation.

The histological alterations were not extended enough to determine a significant decrease in the relative weight of these immune organs (Table 5), as also reported in some of the previously published studies (Kubena et al., 1998; Rashidi et al., 2020; Zabiulla et al., 2021; Alharthi et al., 2022). However, in all likelihood, they caused disruption of the normal microenvironment required for T and B cell development and functionality. Indeed, our findings showed that, on the last day of the feeding trial (i.e. d 35), broilers fed AFB_1-contaminated diet (G2) had significantly ($P < 0.05$) lower Ab titers against all of the vaccinal viruses considered (ND, H5N1 and H9N1) as compared with broilers fed control diet (G1) (Table 6). A significant ($P < 0.05$) decrease in Ab titers against ND and H5N1 viruses was also observable at earlier time points,

Table 6. Effects of hydrated sodium calcium aluminosilicate (HSCAS) on antibody titers against Newcastle disease virus (ND) and avian influenza type A viruses (subtypes H5N1 and H9N1) in broiler chickens experimentally challenged with aflatoxin B_1 (AFB_1) for 35 d.

		Dietary treatments			
Variables		Control	AFB_1	AFB_1 + HSCAS	*P*-value
7 d	ND	3.33 ± 0.4	3.83 ± 0.4	3.5 ± 0.2	0.147
	H5N1	3.66 ± 0.3^{b}	3.13 ± 0.3^{c}	5.83 ± 0.4^{a}	0.032
	H9N1	6.83 ± 0.4	7.5 ± 0.2	7.5 ± 0.4	0.178
14 d	ND	1.5 ± 0.3^{b}	1.16 ± 0.3^{c}	2.5 ± 0.3^{a}	0.031
	H5N1	2.52 ± 0.2^{a}	2.03 ± 0.3^{b}	2.33 ± 0.2^{a}	0.038
	H9N1	4.83 ± 0.7	4.0 ± 0.5	3.66 ± 0.4	0.329
21 d	ND	4.83 ± 0.13^{a}	4.13 ± 0.14^{b}	4.96 ± 0.14^{a}	0.046
	H5N1	1.85 ± 0.13^{b}	1.33 ± 0.13^{c}	2.16 ± 0.13^{a}	0.028
	H9N1	2.59 ± 0.14	2.45 ± 0.14	2.73 ± 0.16	0.113
28 d	ND	4.75 ± 0.11^{a}	4.02 ± 0.11^{b}	4.93 ± 0.12^{a}	0.041
	H5N1	1.92 ± 0.11^{b}	1.42 ± 0.12^{c}	2.31 ± 0.11^{a}	0.032
	H9N1	2.09 ± 0.10	2.04 ± 0.11	2.03 ± 0.11	0.353
35 d	ND	5.16 ± 0.7^{a}	4.33 ± 0.4^{b}	5.59 ± 0.13^{a}	0.042
	H5N1	3.16 ± 0.17^{a}	2.33 ± 0.15^{b}	3.5 ± 0.16^{a}	0.037
	H9N1	1.54 ± 0.13^{b}	1.16 ± 0.14^{c}	2.16 ± 0.13^{a}	0.013

Data (IU) are means (± SEM) for 40 chickens per group (5 chickens from each replicate).

Within the same row, significant differences at $^{*}P < 0.05$ are indicated by different superscript letters (a, b, c), whereas non-significant differences are indicated by the same superscript letters or without any letters.

particularly starting from d 7 for anti-H5N1 virus titers, and starting from d 14 for anti-ND virus titers. Conversely, there was no effect of AFB_1 on the production of Ab directed against H9N1 virus until d 35, possibly due to the vaccine programme used. Taken together, these data suggest that under the influence of AFB_1, the immune function of the broiler chickens, particularly their humoral immune response, was impaired. Similar to our study, the association between aflatoxicosis and decreased Ab titers against ND virus in broiler chickens has been reported by Rahim et al. (1999), Oğuz et al. (2003) and Hassan et al. (2021). Other authors, instead, reported different results in this respect, namely no change in Ab titers against ND virus (Rashidi et al., 2020; Zabiulla et al., 2021). Our data regarding the association between aflatoxicosis and decreased Ab titers against AI viruses are consistent with the results of Rashidi et al. (2020).

Influence of HSCAS on the Response of Broiler Chickens to AFB_1

As discussed above, no appreciable impairment of nutrient digestibility was observed in the chickens of our study in response to AFB_1. In light of this, the present investigation disappointingly failed in its original aim to assess the ability of HSCAS to mitigate the negative impact of aflatoxicosis on this specific aspect of chicken physiology. However, the DMD and PR in the chickens receiving the AFB_1-contaminated diet supplemented with HSCAS (G3) were significantly higher ($P < 0.05$) than in chickens fed the non-supplemented AFB_1-contaminated diet (G2), and also showed tendency towards higher values than in chickens fed the control diet (G1) (Table 3). This finding suggests a scenario in which the mycotoxin challenge, in actual fact, exerted some "subtle" negative influence on these specific digestibility-related parameters (i.e. not severe enough to cause significant impairment), against which HSCAS was fully protective. Future investigations carried out under more suitable experimental conditions will allow us to assess whether HSCAS is also effective at protecting against more pronounced AFB_1-induced alterations of intestinal digestive functions, just like it was demonstrated in previous studies for other aluminosilicate-based mycotoxin binders such as bentonite, zeolite, and smectite (Liu et al., 2018; Alharthi et al., 2022).

As for those aspects of chicken physiology that this study found to be appreciably affected by AFB_1-exposure (namely, liver and kidney physiology, glucose homeostasis, fat deposition, immune system physiology, growth), our results revealed that dietary HSCAS supplementation (0.2%), depending on the specific aspect (and parameter) investigated, was able to ensure either partial or complete protection from the alterations induced by AFB_1, and even lead, in some cases, to improvements over the control (non-aflatoxicated) condition. With respect to these effects of HSCAS, the present study provides knowlegde that is in general agreement with the published literature (Oğuz et al., 2000; Pimpukdee et al., 2004), but also includes new original observations.

Starting from the liver-related aspects, this study observed that dieraty HSCAS exerted complete protection against the hepatic injury induced in chickens by AFB_1. In the first place, this is indicated by the finding that the AFB_1-challenged broiler chickens fed the HSCAS-supplemented diet (G3) had an almost normal histomorphological appearance of the liver, in which the alterations detected in the AFB_1 intoxicated group (G2), namely hepatocellular injury, necrosis, fibrosis, inflammation, and bile duct hyperplasia, were considerably mitigated or even no more detectable (Figure 1c). Moreover, and consistent with this, the AFB_1-challenged broiler chickens fed the HSCAS-supplemented diet (G3) also had serum levels of ALT enzyme activity that were significantly lower ($P < 0.05$) than those measured in the non-supplemented aflatoxicated broilers (G2) (Table 4). Other studies in the published literature, using HSCAS and AFB_1 at levels and relative proportions comparable to those adopted herein, have likewise demonstrated the capacity of HSCAS to prevent the structural damage induced by AFB_1 to the liver tissue of broiler chickens (Ledoux et al., 1998; Zhao et al., 2010; Hassan et al., 2021). However, it must be pointed out that, in our study, the ALT activity of the AFB_1-challenged and HSCAS-

supplemented chickens (G3) was decreased to levels that were also significantly lower ($P < 0.05$) than those measured in control chickens (non-challenged and nonsupplemented chickens) (G1) (Table 4). In addition, a similar significant decrease below the control levels was recorded for the serum AST activity of the chickens fed AFB_1-contaminated and HSCAS-supplemented diet (G3) ($P < 0.05$ vs. G1) (Table 4), despite this parameter was among those not affected by the mycotoxin challenge.

Taken together, these findings suggest that, under the experimental conditions of the present study, there was some additional hepatotoxic factor in the basal diet to which all chickens were exposed (independently of the experimental group), and which was somehow sensitive to the neutralizing action of HSCAS. The identity of this factor acting as an additional source of hepatic damage, and the mechanism of its interaction with HSCAS remain unknown. Some suspicions might fall on the AFB_1 detected in the basal commercial feed used to prepare the three experimental dietary treatments tested in this study. However, the basal contaminating level of AFB_1 measured was very small (1 ppb), and well below the maximum level of AFB_1 permitted in broiler feeds by most countries for safeguarding chickens from well-being and health dangers (e.g. 20 ppb in the European Union and 10-20 ppb in China) (Liu et al., 2018; Alharthi et al., 2022; Xu et al., 2022). Another possibility to be considered is suggested by the results of previous studies, that showed significantly up-regulated expression of genes encoding for antioxidant enzymes (catalase and superoxide dismutase) in the liver of chickens receiving dietary supplementation with 0.5% HSCAS (in the absence of AFB_1-exposure) (Chen et al., 2014). So, a more likely scenario may be that the HSCAS ingested by the chickens of our study, besides trapping and neutralizing the AFB_1 experimentally added to the feed, also improved the liver antioxidant capacity, increasing the organ's ability to cope with some additional, "naturally present" and oxidative stress-inducing agent, to which the animals were presumably exposed through the basal diet. Further investigations are needed to verify these hypotheses. However it is interesting to note that, in exerting this beneficial influence on the hepatic enzyme activities of broilers exposed to AFB_1 in our study, HSCAS behaved similarly to the poultry litter biochar and the lactic acid bacteria that Rashidi et al. (2020) and Liu et al. (2018), respectively, tested for their ability to prevent or mitigate aflatoxicosis induced by lower doses of AFB_1 (0.5 ppm and 40 ppb, respectively). Moreover, HSCAS in our study performed better than the smectite-based binder tested by Zabiulla et al. (2021), which proved completely unable to prevent increases in AST activity induced by 0.5 ppm of AFB_1, as well as better than the bentonite and zeolite binders tested by Alharthi et al. (2022), which showed only partial and very limited protection against the increases in AST and ALT activities induced by 0.25 ppm of AFB_1. Of course, it should be kept in mind that the validity of these comparisons is somewhat limited by the fact that the experimental conditions under which the various mycotoxin binders have been tested are not exactly the same (with respect to one or more of the following aspects: bird strain, duration of the treatment, basal diet composition, source of AFB_1, presence or absence of other contaminating mycotoxins in the diet, and so on).

Consistent with a liver tissue integrity that was not only preserved, but even improved compared to the control condition, the AFB_1-exposed broiler chickens receiving HSCAS supplementation showed signs of improved hepatic functionality, particularly in terms of lipid metabolism. This was indicated by the measurement in these chickens (G3) of serum levels of total cholesterol that were not only lower than those measured in their nonsupplemented counterparts (G2) ($P < 0.05$), but also lower than those measured in control chickens (G1) ($P < 0.05$) (Table 4). The mitigation of cholestasis probably played a contributing role to the lowering of cholesterol levels down to control values, but the further decrease of cholesterol levels below control values was more likely determined by a more efficient hepatic control of cholesterol metabolism resulting from HSCAS-mediated neutralization of the additional hepatotoxic factor present in basal diet. To the best of our knowledge, this is the first report to document the ability of HSCAS to prevent and reverse the AFB_1-induced

increases in total cholesterol. A similar outcome was reported by Liu et al. (2018) for smectite and lactic acid bacteria.

Another finding of our study related to the hepatic functionality of the AFB_1-challenged chickens fed the HSCAS-supplemented diet was that the serum TP levels in these birds (G3) were significantly higher than those measured in the aflatoxicated chickens (G2) ($P < 0.05$), with tendency towards increased values as compared to control chickens (G1) (Table 4). Based on the scenarios that have been outlined above, this response pattern suggests that the protein synthesis function of the chickens' liver: (a) differently from the cholesterol-regulating one, was not affected by the additional hepatotoxic agent present in the basal diet; (b) similarly to some digestibility parameters (DMD, PR), was only subtly affected by the mycotoxin challenge (to an extent that was not severe enough to cause significant decreases in the serum TP levels of the aflatoxicated birds); (c) was fully protected against this subtle negative influence of AFB_1 by HSCAS, which allowed the hepatic protein synthesis function to take place normally. Numerous studies in the published literature demonstrate that HSCAS (used at levels ranging from 0.2 to 1%) can also be effective at providing partial (Kubena et al., 1990; Huff et al., 1992; Abo-Norag et al., 1995; Kubena et al., 1998; Hassan et al., 2021) or complete (Ledoux et al., 1998; Zhao et al., 2010) protection in situations in which chicken hepatic protein synthesis shows clear-cut sensitivity to AFB_1, resulting in significantly decreased serum levels of TP.

In our study, HSCAS completely counteracted the AFB_1-induced increase in serum levels of uric acid. This was indicated by the finding that AFB_1-challenged and HSCAS-supplemented chickens (G3) had serum levels of uric acid not different from those recorded in the control chickens (G1) (Table 4). As already explained in the previous part of this discussion, this result may reflect either the efficacy of HSCAS at protecting the hepatic control of protein and amino acid metabolism from the derangement caused by AFB_1 or, and more likely, the efficacy of HSCAS at protecting the kidneys from the negative influence of AFB_1 on their excretory function. In counteracting the AFB_1-induced increase in uric acid, HCSAS seemed to perform in our study slightly better than in the study by Hassan et al. (2021), where it provided substantial but not complete protection on this parameter. On the other hand, always considering the limitations that are inherent to between-studies comparisons, the protective efficacy of HSCAS documented herein in relation to this specific parameter seems more similar to that reported for 2 other anti-mycotoxin additives (a toxin binder and the poultry litter biochar) by Rashidi et al. (2020). In any case, it must be acknowledged that the measuremet of uric acid alone provides only partial information regarding the health status of the chickens' kidneys, and thus only partial information regarding the efficacy of HSCAS at protecting these organs from AFB_1-induced damage. In this regard, some earlier studies can be mentioned that found HSCAS to be only partially able (at 0.5%) or unable (at 0.25%) to prevent increases induced by AFB_1 (at 1 mg/kg and 5 mg/kg, respectively) in other serum biochemical markers of renal functionality (creatinine and urea nitrogen, respectively) (Kubena et al., 1998; Hassan et al., 2021).

In relation to the alterations that AFB_1 was found to induce in our study on serum glucose levels and relative weight of abdominal fat, dietary HSCAS provided only partial protection. This was indicated by the finding that the chickens fed the AFB_1-contaminated and HSCAS-supplemented diet (G3) had serum glucose levels (Table 4) and relative abdominal fat weight (Table 5) significantly less increased than in the non-supplemented aflatoxicated birds (G2) ($P < 0.05$), but still significantly higher than in control birds (G1) ($P < 0.05$). This result provides further support to the hypothesis formulated above that the increased glucose and increased relative weight of abdominal fat observed in our aflatoxicated broilers may be interrelated, and may both depend on a perturbation induced by AFB_1 in the endocrine pancreas of the chickens (Ko et al., 2021), rather than in the liver. In view of this scenario, the fact that the protection exerted by HSCAS on the two parameters was only partial, suggests that - under the experimental conditions of the present study - the chickens' endocrine pancreas was probably more sensitive than other

organs (such as the liver) to AFB_1 toxicity. In other terms, the amount of feed-contaminating AFB_1 that HSCAS fails to trap is probably too small to cause detectable damage to organs such as the liver, but it is still large enough to cause detectable damage to the pancreas. To the best of our knowledge, this is the first report to document the ability of HSCAS to protect against AFB_1-induced increases in glucose levels. As for the efficacy of HSCAS at preventing increases in abdominal fat, HSCAS in our study performed less well than two other anti-mycotoxin additives (a toxin binder and the poultry litter biochar) tested by Rashidi et al. (2020), that instead were found to exert complete protection on this parameter. However, it should be considered that, in the latter study, the chickens were exposed to a lower level of AFB_1 (0.5 ppm), and the extent to which abdominal fat increased in the aflatoxicated animals was smaller than in the present study. Moreover, the possibility cannot be ruled out that other differences existing in the experimental conditions adopted in the present study and in that by Rashidi et al. (2020) (e.g. treatment duration, AFB_1 source, basal diet composition) may have played an influencing role on the protective performance of the different binders.

As concerns the immune-related aspects that AFB_1 negatively influenced in this study, dietary supplementation of HSCAS was found to ensure complete protection, leading to a substantially preserved, and even improved immune function. This was indicated by the finding that, on d 35, the Ab production against all of the viruses considered (ND, H5N1 and H9N1 viruses) was significantly higher ($P < 0.05$) in the AFB_1-challenged chickens receiving the HSCAS-supplemented diet (G3) than in their nonsupplemented counterparts (G2), and showed values that were not different from (ND, H5N1), or significantly higher than (H9N1) those recorded in the chickens fed control diet (G1) (Table 6). The protective effect of HSCAS on the chickens' humoral response to ND and H5N1 viruses was also observed at earlier time points (particularly, starting from d 14 for anti-ND virus titers and starting from d 7 for anti-H5N1 virus titers), whereas the protective/enhancing effect on the Ab response to H9N1 virus was not observed until d 35 (Table 6), possibly due once again to a background influence of the vaccine programme used.

In addition to this normal (or higher than normal) Ab production, the broilers fed the AFB_1-contaminated and HSCAS-supplemented diet also showed a substantially preserved histomorphological appearance of the lymphoid organs thymus and bursa, which was indicative of mitigation of the AFB_1-induced tissue damage. Particularly, in the thymus gland of these chickens (G3) (Figure 1c1), restored lymphocyte population, improved cortex and medulla organization, and reduced connective tissue deposition were observed, relative to the thymus of the non-supplemented aflatoxicated chickens (G2). Similarly, the histological structure of the bursa in G3 showed substantially improved features, with almost normal architecture (Figure 1c2).

It is of note that, in the AFB_1-challenged chickens receiving the HSCAS-supplemented diet (G3), the relative weights of all immune organs (thymus, bursa and spleen) were found to be significantly higher than in the nonsupplemented aflatoxicated chickens (G2) ($P < 0.05$), showing values not different from (and even numerically higher than) those recorded in control chicks (G1) (Table 5). As commented above about some of the digestibility- (DMD and PR) and liver- (serum TP) related parameters, this finding suggests that the mycotoxin challenge probably had some "subtle" negative impact on the development of the chickens' immune organs (i.e. not severe enough to result in significant reduction of their relative weights), and that dietary supplementation with 0.2% HSCAS was fully protective against it, allowing normal immune organ development. However, based on the findings of an earlier study (Kubena et al., 1990), the possibility exists that dietary supplementation of HSCAS, even at higher inclusion levels (0.5%) may not be able to exert any protection against a significant decrease in bursa relative weight induced by higher doses of AFB_1 (7.5 mg/kg).

The efficacy that HSCAS has shown in our study at preventing the AFB_1-induced depression of the chickens' immune response to the vaccinal ND virus has also been reported in previous studies (Rahim et al., 1999; Hassan et al.; 2021). However, it is worth noting that in the

latter, HSCAS was found to exert only partial protection on this response (leading to Ab titers that were higher than in aflatoxicated chickens, but still lower than in control chickens), and this occurred despite the use of higher dietary inclusion levels of HSCAS (0.3 and 0.5%, respectively) and lower feed-contaminating levels of AFB_1 (0.5 and 1 ppm, respectively) than in the present study. As for the efficacy of HSCAS in preventing the AFB_1-induced depression of the chickens' immune response to vaccinal AI viruses, it is documented by the present study for the first time. In exerting this protection, HSCAS seems much superior to the mycotoxin binder tested by Rashidi et al. (2020), which proved completely unable to prevent the adverse effects of 0.5 ppm AFB_1 on Ab titers against AI virus. Finally, the efficacy of HSCAS at substantially mitigating the AFB_1-induced changes in the histomorphology of thymus and bursa, is similarly described here for the first time. In this respect, HSCAS may even be superior to the smectite-based mycotoxin binder tested by Zabiulla et al. (2021), considering that the latter binder was used at the same inclusion level as HSCAS in our study (0.2%), but to counteract a lower feed contaminating level of AFB_1 (0.5 ppm). Once again, such comparisons should be approached with caution, due to differences in the experimental conditions under which the behavior of the various mycotoxn binders has been studied.

The last point to be discussed is the protective efficacy of dietary HSCAS supplementation against the growth performance impairment produced in the chickens by dietary exposure to AFB_1. In this respect, our study revealed that dietary HSCAS was not only able to preserve the growth of the chickens in the face of the negative impact of the mycotoxin challenge, but also improved it over the control condition. Indeed, the inclusion of 0.2% HSCAS in the AFB_1-contaminated diet (G3) significantly increased the values of all weight-related parameters (final BW, BWG, ADWG) ($P < 0.05$) and significantly decreased the value of FCR ($P < 0.05$) in comparison with non-supplemented AFB_1-contaminated diet (G2), leading to values that were significantly higher (final BW, BWG, ADWG) ($P < 0.05$) or significantly lower (FCR) ($P < 0.05$) than those measured in chickens fed the control diet (G1) (Table 2).

There are various studies in the published literature documenting the ability of HSCAS (at levels ranging from 0.5% to 1%) to completely prevent the growth inhibitory effects of AFB_1 (1−4 mg/kg) on broiler chickens, keeping productivity indices at control levels (Abo-Norag et al., 1995; Ledoux et al., 1998; Hassan et al., 2021). However, to the best of our knowledge, this is the first study reporting significant improvements in all of the aflatoxin-sensitive growth-related parameters (final BW, BWG, ADWG, and FCR) over the control condition. In Hassan et al. (2021), such an effect was observed only for FCR.

Based on what was discussed above, it is interesting to point out that our finding of improved growth performance in AFB_1-challenged and HSCAS-supplemented chickens: (a) was recorded despite the persistence in these birds of some degree of metabolic alterations (elevated serum glucose levels and increased relative abdominal fat weight), and in all likelihood (b) reflected the improvements occurring in the structural integrity and functional performance of the liver (serum levels of ALT, AST, total cholesterol), as well as in the efficiency of some immune functions (Ab production in response to H9N1 vaccine). Therefore, from a mechanistic point of view, this improvement in the growth performance parameters might be related to some additional properties of HSCAS, that besides neutralizing the experimental challenge with AFB_1, would also neutralize some other hepatotoxic (and possibly immunotoxic) factor present in the basal diet, by direct interaction with it (i.e. acting as a binder), or by strengthening the capacity of hepatocytes (and possibly immune cells) to cope with it (i.e. acting as an inducer of antoxidant enzyme expression) (Chen et al., 2014). The inclusion in our study design of a fourth experimental group receiving HSCAS without AFB_1 would have helped verify this hypothesis. In this regard, it is worth mentioning that some Authors reported significantly increased values (Phillips et al., 1988; Kubena et al., 1990), or tendency towards increased values (Chen et al., 2014) for final BW and/or WG in broiler

chickens fed diets containing HSCAS alone, in comparison with the control animals; however, they did not discuss this result.

CONCLUSIONS AND APPLICATIONS

1. The present study adds to previous research in demonstrating the deleterious impact that relatively high feed contaminating levels of AFB_1 (2.5 ppm) can have on both the health and productive performance of growing broiler chickens, as well as the effective protection that dietary supplementation with the aflatoxin-binder HSCAS, at the inclusion level of 2 g/kg feed (0.2%), can safely ensure against the toxicity of this challenge.
2. In addition, the original or unusual observations made during the realization of this study regarding the response of broiler chickens to the mycotoxin challenge and the influence exerted by HSCAS on this response, as well as on basal chicken physiology, support the concept that the sensitivity of each single production- and health-related parameter to the detrimental influence of AFB_1 and the protective potential of HSCAS can vary, depending on a wide and complex array of interrelated factors that likely include, among others, the levels of AFB_1 to which the animals are exposed, the levels of HSCAS that are incorporated in the diet, the overall duration of AFB_1 exposure and HSCAS supplementation, the composition of the basal diet (also in terms of possible chemical contaminants).
3. On the whole, this study brings new knowledge regarding the potential manifestations of aflatoxicosis in broiler chickens and spectrum of HSCAS' beneficial effects, thereby providing a contribution towards improved identification and control of an aflatoxin problem in the poultry industry.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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