Effects of aflatoxins and fumonisins, alone or in combination, on performance, health, and safety of food products of broiler chickens, and mitigation efficacy of bentonite and fumonisin esterase

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Abstract

The current study evaluated the effects of feeding diets contaminated with aflatoxin B1 (AFB1), fumonisins (FBs), or both, on the performance and health of broiler chickens and the safety of their food products, as well as the efficacy of bentonite and fumonisin esterase to mitigate the effects of these mycotoxins under conditions representative for sub-Saharan Africa (SSA). Four hundred one-day-old Cobb 500 broiler chickens were randomly assigned to 20 treatments with either a control diet, a diet with moderate AFB1 (60 µg/kg feed) or high AFB1 (220 µg/kg feed), or FBs (17,430 µg FB1+FB2/kg feed), alone or in combination, a diet containing AFB1 (either 60 µg/kg or 220 µg/kg) and/or FBs (17,430 µg FB1+FB2/kg) and bentonite or fumonisin esterase or both, or a diet with bentonite or fumonisin esterase only. The experimental diets were given to the birds from day 1 to day 35 of age and the effects of the different treatments on production performance were assessed by feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR). Possible health effects were evaluated through blood biochemistry, organ weights, mortality, liver gross pathological changes and vaccine response. Residues of aflatoxins (AFB1, B2, G1, G2, M1 and M2) were determined in plasma, muscle and liver tissues using validated UHPLC-MS/MS methods. The results obtained indicated that broiler chickens fed high AFB1 alone had poor FCR when compared to a diet with both high AFB1 and FBs ($p = 0.0063$). Serum total protein and albumin from birds fed FBs only or in combination with moderate or high AFB1 or detoxifiers increased when compared to the control ($p < 0.05$). Liver gross pathological changes were more pronounced in birds fed contaminated diets when compared to birds fed the control or diets supplemented with mycotoxin detoxifiers. The relative

weight of the heart was significantly higher in birds fed high AFB1 and FBs when compared to the control or high AFB1 only diets ($p < 0.05$), indicating interactions between the mycotoxins. Inclusion of bentonite in AFB1-contaminated diets offered a protective effect on the change in weights of the liver, heart and spleen ($p < 0.05$). Residues of AFB1 were detected above the limit of quantification (max: 0.12 ± 0.03 µg/kg) in liver samples only, from birds fed a diet with high AFB1 only or with FBs or the detoxifiers. Supplementing bentonite into these AFB1-contaminated diets reduced the levels of the liver AFB1 residues by up to 50%. Bentonite or fumonisin esterase, alone, did not affect the performance and health of broiler chickens. Thus at the doses tested, both detoxifiers were safe and efficient for use as valid means of counteracting the negative effects of AFB1 and FBs as well as transfer of AFB1 to food products (liver) of broiler chickens.

Key words: Aflatoxins; broiler chickens; bentonite; co-contamination; fumonisin esterase; fumonisins; Kenya; sub-Saharan Africa; feed additives; food safety

Introduction

Quality and safety of feeds are determined by, among other factors, contamination by fungal secondary metabolites known as mycotoxins. Aflatoxins (AFs) and fumonisins (FBs) (Supplementary Figure S1 and S2) are the major mycotoxins of concern in sub-Saharan Africa (SSA) due to their widespread occurrences in crops and adverse effects on both animal and human health [1]. Aflatoxins are produced mostly by *Aspergillus flavus* and *A. parasiticus*. Poor storage coupled with warm and humid weather conditions in SSA enhances contamination of agricultural products by AFs. In addition, lack of awareness and laxity in enforcing regulatory laws have resulted in extensive contamination of food and feed by mycotoxins [2]. Among the different AFs that have been reported, aflatoxin B1 (AFB1) is the most prevalent and has the highest toxicity in both animals and humans [3]. According to various studies conducted in SSA, AFs were present in over 60% of poultry feeds and levels above 1,000 µg/kg were occasionally reported in some studies [4]. In poultry, AFs have been associated with reduced growth, organ damage, immunosuppression, and increased mortality, causing great economic losses [5].

Fumonisins are secondary metabolites of *Fusarium* species, mainly *Fusarium verticillioides* and *F. proliferatum*. Though considered prevalent in temperate regions, previous studies demonstrated that they are also major contaminants of food and feed in SSA [1,4]. Among the various FBs reported, fumonisin B1 (FB1) (Supplementary Figure S2) is the most toxic and prevalent worldwide [6]. Poultry are considered to be more resistant to FBs toxicity compared to pigs and horses and an increase in mortality was only seen during the first 3 weeks of their life due to dietary FB1 levels above 125 mg/kg [7]. However, with the improvement in the performance of modern broilers and the move towards antibiotic-free production, even low to moderate levels of FBs have been reported to negatively affect animal health [8]. Levels below the maximum guidance values set by the United State of America (USA) and the European Union (EU) in feed and close to SSA field conditions (ranging from 10 mg/kg to 20 mg/kg feed) were shown to affect immunological and metabolic functions resulting in increased susceptibility to infectious diseases in poultry [9]. Fumonisin-contaminated feeds were further linked to reduced feed intake (FI), reduced body weight gain (BWG), and diarrhoea in poultry [8,10].

Co-contamination of poultry feeds results from feed ingredients being contaminated by different mycotoxin-producing fungi and the ability of certain fungi to produce more than one mycotoxin [4]. This co-contamination is of great concern as mycotoxins can interact with each other, resulting in adverse effects even at low levels of exposure [11]. In SSA, the co-contamination of poultry feeds by AFs and FBs was the most commonly reported combination [4,12]. A combination of AFB1 and FBs resulted in pronounced poor growth, changes in blood biochemistry and liver histopathology of broiler chickens [13].

In addition to having negative effects on the health and productivity of animals, carry-over of AFs and FBs to animal-derived food products has been observed. Although in general a limited transfer of AFs was reported, intake of low levels of AFs over a long period could lead to detrimental health effects to human consumers [14,15]. Aflatoxin B1 has been detected in broiler chickens' liver and gizzard samples collected from abattoirs and markets [16]. In feeding trials, dietary AFB1 (50 to 100 μg/kg feed) resulted in broilers' liver AFB1 residues of about 1 μg/kg [15]. Regarding FBs, recent investigations suggested that FBs could

accumulate in liver and muscle tissues even when present in feed at low concentrations of between 7.5 and 20 mg/kg [17,18]. No information is currently available on the carry-over ratios in case of co-contamination of AFs and FBs.

Concerning post-harvest mitigation strategies, the use of mycotoxin detoxifiers, both binders and modifiers, are considered an efficient way to reduce the negative effects of mycotoxins on animal health and productivity. Mycotoxin binders including bentonite (BENT), hydrated sodium calcium aluminosilicates (HSCA), clinoptilolite, and zeolite have been investigated for their ability to reduce the negative impacts of AFs on chicken health and productivity [19,20]. Negative effects of *Fusarium* mycotoxins such as FBs were however not alleviated by mycotoxin binders [21]. More recently, fumonisin esterase (FZYM) which is a mycotoxin modifier and an enzyme produced by *Komagataella phaffii* DSM 32159 was reported to be capable of cleaving the side chains of FB1 with the formation of fully hydrolysed HFB1 and partially hydrolysed pHFB1a and pHFB1b, which are less toxic than the parent FB1 (Supplementary Figure S2) [22]. Both BENT and FZYM have been declared safe by the European Food Safety Authority and are approved by the European Commission for use in poultry, ruminants, and pigs [23,24]. The BENT and FZYM are commercialized as Mycofix® Secure and FUM*zyme*®, respectively (by Biomin® GmbH, part of DSM) according to Regulation (EC) No 1831/2003 [25], and are included in the EU register of feed additives [26]. However, their combined application in the case of co-contamination of broiler chicken feed has not been studied before. In addition, safety and efficacy of these mycotoxin detoxifiers are often not evaluated under experimental conditions similar to rearing conditions in SSA including mycotoxin levels, temperature, veterinary control systems and feed composition, among other factors.

Evaluation of the safety and efficacy of mycotoxin detoxifiers in poultry can be conducted by observing changes in growth performance, blood biochemical parameters, vaccine responses, and liver histopathological changes, among other parameters [3,19]. Most *in vivo* chicken studies have used very high levels of AFB1 (2,000 to 5,000 μg/kg feed) to elicit toxicities and evaluate the effectiveness of detoxifiers within very short experimental periods [20,27]. However, these high levels are less likely to be reported in field

conditions, with low to moderate doses of mycotoxins and co-occurrences of mycotoxins being of great concern [4]. Therefore, the present study aimed at evaluating the effects of AFB1 and FBs (FB1 + FB2), alone or in combination, on the health, performance and safety of food products of broiler chickens, as well as the efficacy and safety of BENT and FZYM in reducing these effects under mycotoxin doses and farming practices representative for SSA. In our previous multi-mycotoxin study of poultry and dairy cattle feeds and feed ingredients from Kenya, AFB1 and total FBs at max. levels of 99 µg/kg and 14 mg/kg, respectively, were reported [1].

Materials and methods

Ethical approval and mycotoxins

This study was conducted at the International Livestock Research Institute (ILRI), Nairobi, Kenya. All animal experimental procedures and protocols were reviewed and approved by the ILRI's animal care and use ethical committee (approval IACUC-RC2019-03).

Aflatoxins and FBs used in this study were produced by inoculating maize with *A. flavus* and *F. verticillioides* strains, respectively, as described by Ochieng et al. [28]. The fungal strains were supplied by the Mycology and Mycotoxin Laboratory, University of Nairobi, Kenya. These strains had been isolated in previous studies and were high producers of AFs or FBs [29,30]. The culture materials were analysed for major AFs and FBs using LC-MS/MS method [31]. Cultures inoculated with *A. flavus* contained 66,419 µg AFB1/kg substrate and 1,586 µg AFB2/kg substrate, whereas maize cultures inoculated with *F. verticillioides* contained 170,258 µg FB1/kg and 169,311 µg FB2/kg.

Experimental diets and treatment groups

Basal diets with no antibiotics, coccidiostats or growth promoters and formulated according to the National Research Council to meet nutrient requirements for starter and grower chickens [32], were obtained from a commercial supplier and used as control diet. The proximate composition of the control diet is shown in Supplementary Table S1. Mycotoxin contamination of the control diet was investigated using a LC-MS/MS

method as described by Sulyok et al. [33]. The levels of all tested mycotoxins were below EU regulatory or guidance values [34–36] and have been shown to be non-toxic to poultry in other studies [15,37,38]. Concentrations of AFB1 (0.4 and 0.8 µg/kg), FB1 (18.0 and 78.4 µg/kg) and FB2 (7.2 and 30.4 µg/kg) were detected in the control starter and grower feeds, respectively (Supplementary Table S1).

To obtain the treatment diets contaminated with AFB1 or FBs (FB1+FB2), or both, maize culture materials were incorporated into 5,000 g of control diet to make a premix. This premix was further incorporated into feed quantities necessary for the trials to provide AFB1 (60 or 220 µg/kg feed) and FBs (FB1+ FB2) (17,430 µg/kg feed) contaminated diets. The FBs-contaminated diets contained 14,160 µg FB1/kg feed and 3,270 µg FB2/kg feed. The BENT and FZYM were included in relevant diets at levels of 2 g BENT/kg feed and 0.012 g FZYM/kg feed (Biomin® GmbH, part of DSM). The 20 dietary treatments are shown in Table 1.

Table 1: The different treatment diets administrated to broiler chickens from 1 to 35 d of age

Treatment	**FBs** 17,430 µg/kg	**Moderate AFB1** (M AFB1) 60 µg/kg	**High AFB1** (H AFB1) 220 µg/kg	**Bentonite** (BENT)	**Fumonisin esterase** (FZYM)
T1 - Control	-	-	-	-	-
T2 - FBs	+	-	-	-	-
T3 - FBs + FZYM	+	-	-	-	+
T4 - FBs + FZYM + BENT	+	-	-	+	+
T5 – H AFB1	-	-	+	-	-
T6 - H AFB1 + BENT	-	-	+	+	-
T7 - H AFB1 + BENT + FZYM	-	-	+	+	+
T8 - H AFB1 + FBs	+	-	+	-	-

T9 - H AFB1 + FBs + BENT	+	-	+	+	-
T10 - H AFB1 + FBs + FZYM	+	-	+	-	+
T11 - H AFB1 + FBs + BENT + FZYM	+	-	+	+	+
T12 - M AFB1	-	+	-	-	-
T13 - M AFB1 + BENT	-	+	-	+	-
T14 - M AFB1 + BENT + FZYM	-	+	-	+	+
T15 - M AFB1 + FBs	+	+	-	-	-
T16 - M AFB1 + FBs + BENT	+	+	-	+	-
T17 - M AFB1 + FBs + FZYM	+	+	-	-	+
T18 - M AFB1 + FBs + BENT + FZYM	+	+	-	+	+
T19 – FZYM	-	-	-	-	+
T20 – BENT	-	-	-	+	-

M AFB1- Moderate AFB1, H AFB1- High AFB1, BENT- Bentonite, FZYM- Fumonisin esterase, FBs- Fumonisin B1 + Fumonisin B2, AFB1- Aflatoxin B1.

Broiler chickens' management

A total of four hundred unsexed one-day-old broiler chickens (Cobb 500) were bought from a commercial farm and used for the trial from 1 to 35 d of age. The chickens had been vaccinated against infectious bursal disease (IBD), Newcastle disease (NCD) and Infectious Bronchitis (IB) (Cevac® Transmune IBD and Cevac® Vitabron L, both from Ceva Intertropical Africa, Nairobi, Kenya) administered through spraying on day 1 at the hatcheries. The birds were individually weighed, wing-banded and randomly assigned to 20 treatment groups as shown in Table 1 (20 birds/treatment). Each treatment had four replicates consisting of five birds per replicate housed in a 2-metre square pen with concrete floor and litter (pine wood shavings). The pen walls were made of wire mesh and separated by plywood so that there was no physical contact between the different groups. The pens were cleaned with Hy-Protectol® disinfectant (HighChem, Nairobi, Kenya) before placing the chickens. For the three-week brooding period, heat was provided with infrared

heating lamps placed in each pen. The vaccine routine was administered according to the broiler birds' supplier recommendation at day 14 of age and included a combined NCD and IB vaccine (Combivac C®, Jovac, Amman, Jordan).

The broilers were monitored twice daily for general flock conditions and in case of a mortality, post mortem examination was conducted to determine the cause of the death.

Sampling and sample analysis procedures

Body weight gain, feed intake, and feed conversion ratio

The body weights of all live chickens were measured individually on day 1, 7, 14, 21, 28, and 35. Feed intake (FI) was determined weekly for each pen by subtracting the quantity left from the quantity of feed provided and correcting for mortalities. Body weight gain (BWG) for each bird was calculated by subtracting the final weight from the initial weight. The feed conversion ratio (FCR) was calculated by dividing the FI by BWG [11].

Serum samples for vaccine response tests and biochemical analyses

The birds were vaccinated against NCD and IB disease on day 14 of the trial through drinking water. About 2 mL of blood samples were collected aseptically through the wing vein from the same birds (2 birds/pen) on day 13, 21 and 35 of the feeding period using 23G needle (0.65mm x 30mm) and 2 mL syringe and transferred into serum tubes. The blood samples were centrifuged at 2,500 rpm for 10 min at +4°C and the sera obtained were kept in cryovials at -20°C until analysis. The antibody titres against NCD were determined using hemagglutination inhibition (HI) test [39].

The serum sample collected from each bird on day 35 was divided into two vials for the vaccine response test and the biochemical test. Total protein (TP), albumin (ALB), gamma-glutamyl transferase (GGT), creatinine (CREAT), and uric acid (UA) concentrations were determined using an automated Cobas C600 biochemical analyser (Roche Ltd, Horiba-ABX, Montpellier, France) according to the manufacturer's

recommended procedures. Total antioxidant status (TAS) was evaluated using TAS assay kit (Randox Ltd, Crumlin, United Kingdom). Serum globulin (GLB) level was calculated by subtracting ALB from the TP content [40].

Euthanasia and collection of plasma and organs

At the end of the trial (at 35 d of age), feed was withdrawn from the birds 1.5 to 2 h before weighing all the birds individually. A blood sample (approximately 2 mL) was collected from the wing vein from another 2 birds per pen into heparinized blood collection tubes. Plasma was obtained by centrifuging the blood at 2,500 rpm for 10 min at +4˚C and the plasma was stored at -20˚C until analysis of AFs. The birds were then anaesthetized by a combination of intramuscular injection with 3.10 mg/kg body weight (bw) ketamine hydrochloride (Rotexmedica GmbH, Trittau, Germany) and 0.2 mg/ kg bw midazolam (Troikaa Ltd, Gujarat, India), followed by intravenous injection with 86 mg/kg bw pentobarbital (Bayer, Johannesburg, South Africa). Macroscopic examinations were made on all birds and gross pathological changes in the liver were recorded for 3 birds per pen (12 birds/treatment). The whole liver, spleen, heart, bursa, gizzard and a part of breast muscle (approximately 100 g) were removed from 2 birds per pen (the same birds from which plasma was collected). The organs were weighed and the weights expressed as a percentage of live weight of the birds. The liver and breast muscle samples were then stored at -20˚C within 1 h after sampling. Before AFs residue analysis, the liver and breast muscle samples were minced and homogenized using a Moulinette 320 meat grinder (Moulinex, Barcelona, Spain) and kept at -20˚C until frozen transport for analysis.

Analysis of aflatoxins and their metabolites in plasma, muscle, and liver tissue

The sample pre-treatment and UHPLC-MS/MS analysis were according to the methods developed and validated by De Baere et al. [41] for analysis of AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) in biological matrices from chickens and cattle. The methods were *in-house* validated according to established guidelines [42] and included assessment

of linearity, within- and between-day accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ), extraction recovery, specificity and matrix effect. Blank samples of plasma, liver, and muscle obtained from healthy and untreated chickens were spiked with known concentrations of AFs standards and used to prepare matrix-matched calibration and quality control samples. The LOQ values were between 0.05 - 0.10 ng/mL in chicken plasma; 0.05 - 0.25 µg/kg in chicken muscle and 0.05 - 0.50 µg/kg in chicken liver, depending on the AF. The calculated LOD values ranged between 0.003 and 0.030 ng/mL in chicken plasma; 0.013 - 0.039 µg/kg in chicken muscle and 0.006 - 0.040 µg/kg in chicken liver.

Statistical analysis

The pen was the experimental unit for the analysis of FI and FCR, with starting weight used as a covariate in the analysis using general linear models in R [43] (formula: Response variable ~ Batch + Treatment + Starting weight). The starting weight was included in the model to compensate for any variations at the start of the feeding experiment. The feeding experiment was divided into 2 batches (batch A consisting of 200 birds and batch B also consisting of 200 birds). The batch was included in the model to check for evidence of variations between the batches. For analyses where individual birds were the experimental unit, pen number was included as a random variable in the linear mixed models using the R function lmer from package lme4 (formula: Response variable ~ Batch + Treatment + (1|Pen number)). Pen number was included in the model to compensate for variations in response due to other factors that could not be accounted for in the pens. The 400 birds were housed in 80 pens (5 birds/pen) and the pens labelled 1 to 80. Non-linear data as per Kolmogorov–Smirnov test were first square root transformed before analysis. The responses exhibited by different parameters due to the treatment diets were determined using pre-planned contrasts [44]. Significant differences were considered at the 95% confidence level following a Tukey post hoc test.

For AFs residues in plasma, liver, and muscles, a positive sample was considered as having a concentration above the LOD value while samples below the LOD value were considered negative with no mycotoxin

239 detected. For samples with detectable levels (above LOD) but below LOQ, half the LOQ was used [45]. Carry-
240 over rates from feed into plasma, liver, and meat were expressed as a percentage of the concentration of
241 mycotoxin (µg/kg) in the organ compared to the concentration of the mycotoxin (µg/kg) in the feed x 100
242 [46].

243 **Results**

244 *Production performance*

245 Mortalities of 3 birds from T10, 2 birds each from T5, T9, T14 and T20 and 1 bird each from T1, T3, T4, ,
246 T7, T8, T13, T15, T17 and T19 were recorded during the whole experimental period. According to post-
247 mortem reports, the mortalities were unrelated to dietary treatments. Broiler chickens consuming a diet with
248 high AFB1 alone (T5) or in combination with FBs (T8) had the highest percentage of livers with gross
249 pathological alterations consisting of pale, enlarged and friable livers (83% and 67%, respectively)
250 (Supplementary Figure S3). Over half of the livers from birds that were fed on diets containing FBs
251 supplemented with FZYM (T3) or FBs with both detoxifiers (T4) or a diet containing FBs, moderate AFB1
252 and FZYM (T17) also had pathological changes.

253

254 The FI, BWG, and FCR values at the end of the experimental period (35 days) are shown in Table 2. The
255 different treatments caused non-significant changes on BWG of the broilers ($p > 0.05$). Furthermore, BENT
256 or FZYM alone did not have any effect on BWG and FCR. However, poor FCR was observed in broilers
257 fed high AFB1 only (T5) when compared to those fed both high AFB1 and FBs (T8) ($p = 0.0063$). High
258 AFB1 only or with FBs diets supplemented with BENT or FZYM, or both (T7, T9, T10 and T11) also
259 resulted in poor FCR of the broiler chickens, in contrast to the control diet (T1) or diet with high AFB1 and
260 FBs (T8) ($p < 0.05$). Moreover, moderate AFB1 alone or with FBs diets supplemented with BENT or both
261 BENT and FZYM (T13, T14 and T18) resulted in poor FCR when compared to the control diet (T1) ($p <$

0.05). In comparison to the birds fed the control diet (T1), the FI was significantly higher in broilers fed diets with BENT only (T20) or diets with high AFB1 (T7) or moderate AFB1 alone or with FBs and supplemented with both BENT and FZYM (T14, T17 and T18) ($p < 0.05$).

Table 2: Mean feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) of broiler chickens at the end of the feeding period (35 days). Each treatment group included 20 birds.

Treatment	**FI (g)**	**BWG (g)**	**FCR (g:g)**
T1 - Control	3380[a]	2048	1.53[ab]
T2 - FBs	3590[ac]	1969	1.79[bc]
T3 - FBs + FZYM	3584[ac]	2129	1.75[ac]
T4 - FBs + FZYM + BENT	3630[ac]	2081	1.76[ac]
T5 – H AFB1	3594[ac]	2094	1.81[bc]
T6 - H AFB1 + BENT	3674[ac]	2118	1.79[bc]
T7 - H AFB1 + BENT + FZYM	3937[c]	2076	1.89[c]
T8 - H AFB1 + FBs	3479[ab]	2026	1.47[a]
T9 - H AFB1 + FBs + BENT	3499[ab]	1945	1.84[c]
T10 - H AFB1 + FBs + FZYM	3764[ac]	2053	1.83[c]
T11 - H AFB1 + FBs + BENT + FZYM	3672[ac]	2083	1.78[bc]
T12 - M AFB1	3562[ac]	2017	1.78[bc]
T13 - M AFB1 + BENT	3632[ac]	2063	1.85[c]

Treatment	FI (g)	BWG (g)	FCR (g:g)
T14 - M AFB1 + BENT + FZYM	3896[bc]	2086	1.85[c]
T15 - M AFB1 + FBs	3761[ac]	2102	1.78[bc]
T16 - M AFB1 + FBs + BENT	3808[ac]	2123	1.79[bc]
T17 - M AFB1 + FBs + FZYM	3837[bc]	2031	1.76[ac]
T18 - M AFB1 + FBs + BENT + FZYM	3824[bc]	1896	1.87[c]
T19 - FZYM	3678[ac]	2051	1.77[bc]
T20 - BENT	3874[bc]	2173	1.65[ac]
SEM	145.60	67.50	0.10
Main Effects		***P*-Value**	
FBs	NS	NS	NS
H AFB1	NS	NS	NS
H AFB1 + FBs	NS	NS	NS
M AFB1	NS	NS	NS
M AFB1 + FBs	NS	NS	NS
FZYM	NS	NS	NS
BENT	0.0217	NS	NS
Interactions		***P*-Value**	

Treatment	FI (g)	BWG (g)	FCR (g:g)
FBs vs FBs + FZYM	NS	NS	NS
FBs vs FBs + FZYM + BENT	NS	NS	NS
H AFB1 vs H AFB1 + BENT	NS	NS	NS
H AFB1 vs H AFB1 + BENT + FZYM	NS	NS	NS
H AFB1 + FBs vs H AFB1	NS	NS	0.0063
H AFB1 + FBs vs H AFB1 + FBs + BENT	NS	NS	0.0128
H AFB1 + FBs vs H AFB1 + FBs + FZYM	NS	NS	0.0163
H AFB1 + FBs vs H AFB1 + FBs+ BENT + FZYM	NS	NS	0.0411
M AFB1 vs M AFB1 + FBs	NS	NS	NS
M AFB1 vs M AFB1 + BENT	NS	NS	NS
M AFB1 + FBs vs M AFB1 + FBs + BENT	NS	NS	NS
M AFB1 + FBs vs M AFB1 + FBs + FZYM	NS	NS	NS
M AFB1 + FBs vs M AFB1 + FBs + BENT + FZYM	NS	NS	NS

267 Data are presented as least square means (LSM) and standard error of the mean (SEM) for 20 birds per
268 treatment. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$)

as determined by a Tukey post hoc test. The body weights were measured individually and used to calculate weight gain between the measurements. The feed conversion ratio was calculated by dividing the sum of feed intake by the body weight gain. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite; NS-Not Significant ($p > 0.05$).

Biochemical parameters

Effects of the different experimental diets on serum TP and ALB, concentrations are presented in Table 3. Concentrations of serum TP and ALB differed among the treatments ($p < 0.05$). Serum TP and ALB levels were elevated in broilers fed diets with FBs alone (T2) or in combination with high AFB1 (T8) or moderate AFB1 (T15) when compared to those fed the control diet (T1) ($p < 0.05$). Addition of both BENT and FZYM to diets with FBs alone or with AFB1 (T4, T7, T11 and T17) also increased the broilers serum TP and ALB concentrations in contrast to the control diet ($p < 0.05$). Inclusion of BENT in the diet contaminated with both FBs and high AFB1 (T9) lowered the serum ALB by 15% when compared to the diet with FBs and high AFB1 without BENT (T8) ($p = 0.0223$). In all experimental groups, concentrations of GGT, CREAT, UA, GLB and TAS were not affected ($p > 0.05$) (data not shown). Furthermore, feeding BENT or FZYM alone (T19 and T20) did not change the biochemical parameters.

Weight of organs

The relative organ weights (% of total body weight) of broilers from the different treatments are presented in Table 3. The presence of high AFB1 and FBs (T8) in the diet significantly increased the broilers heart weight by 16% compared to both the control diet (T1) or diet with high AFB1 only ($p < 0.05$). The addition of BENT or FZYM or both to the diet with high AFB1 and FBs (T9, T10, T11) significantly reduced the relative heart weight when compared to AFB1 and FBs diet without the detoxifiers (T8) ($p < 0.05$). Compared to the control diet, the contaminated diets without the detoxifiers (T2, T5, T8, T12 and T15) had no effect on liver, gizzard, spleen and bursa weights ($p > 0.05$). Nonetheless, the spleen weight was significantly increased by 27% when BENT was added to diet with high AFB1 (T6) in comparison to diet

with high AFB1 without BENT (T5) (p = 0.0130). However, the spleen weight was reduced by 23% when both BENT and FZYM was added to the diet with high AFB1 (T7) when compared to the control diet (T1) (p = 0.0104). The liver weights were significantly lower in birds fed high AFB1 supplemented with the two detoxifiers (T7) when compared to high AFB1 without the detoxifiers (T5) or control diet (T1) ($p < 0.05$). Inclusion of FZYM in diets with both moderate AFB1 and FBs (T17) also reduced the liver weights by 13%, in contrast to the control diet (T1) (p = 0.0297). Comparisons of the birds fed the BENT or FZYM only (T19 and T20) and the control diet (T1) did not reveal any significant differences in all the examined organ weights.

Table 3: Effect of different treatments on broiler serum total protein and albumin, and relative weights of liver, spleen, bursa, heart and gizzard (% of total bodyweight) at the end of the trial period (35 days)

Treatment	Total protein (g/L)	Albumin (g/L)	Relative liver weight	Relative spleen weight	Relative bursa weight	Relative heart weight	Relative gizzard weight
T1 - Control	5.07^{a}	3.33^{ab}	1.48^{cd}	0.30^{bc}	0.25	0.69^{ab}	1.11
T2 - FBs	5.68^{d}	3.92^{de}	1.53^{d}	0.29^{bc}	0.30	0.69^{ab}	1.08
T3 - FBs + FZYM	5.38^{ad}	3.74^{bcde}	1.43^{bcd}	0.27^{ab}	0.28	0.70^{b}	1.04
T4 - FBs + FZYM + BENT	5.65^{cd}	4.00^{e}	1.51^{d}	0.30^{bc}	0.28	0.67^{ab}	1.05
T5 - H AFB1	5.17^{ab}	3.55^{acd}	1.46^{cd}	0.26^{ab}	0.30	0.69^{ab}	1.07
T6 - H AFB1 + BENT	5.06^{a}	3.54^{acd}	1.43^{bcd}	0.33^{c}	0.26	0.68^{b}	1.08
T7 - H AFB1 + BENT + FZYM	5.66^{d}	3.87^{ce}	1.23^{a}	0.23^{a}	0.28	0.61^{a}	1.01
T8 - H AFB1 + FBs	5.54^{bd}	3.84^{ce}	1.50^{d}	0.27^{ab}	0.30	0.80^{c}	1.14

Treatment	Total protein (g/L)	Albumin (g/L)	Relative liver weight	Relative spleen weight	Relative bursa weight	Relative heart weight	Relative gizzard weight
T9 - H AFB1 + FBs + BENT	5.10^{ab}	3.26^{a}	1.37^{ad}	0.30^{bc}	0.26	0.70^{b}	1.13
T10 - H AFB1 + FBs + FZYM	5.42^{ad}	3.66^{ae}	1.37^{ad}	0.30^{bc}	0.31	0.71^{b}	1.13
T11 - H AFB1 + FBs + BENT + FZYM	5.39^{ad}	3.79^{ce}	1.51^{d}	0.28^{bc}	0.27	0.67^{ab}	1.09
T12 - M AFB1	5.46^{ad}	3.76^{bce}	1.44^{bcd}	0.28^{ab}	0.25	0.66^{ab}	1.09
T13 - M AFB1 + BENT	5.33^{ad}	3.67^{ae}	1.45^{bcd}	0.28^{ab}	0.27	0.66^{ab}	1.04
T14 - M AFB1 + BENT + FZYM	5.35^{ad}	3.51^{acd}	1.42^{bcd}	0.27^{ab}	0.29	0.66^{ab}	1.04
T15 - M AFB1 + FBs	5.66^{d}	3.83^{ce}	1.44^{bcd}	0.26^{ab}	0.29	0.66^{ab}	1.06
T16 - M AFB1 + FBs + BENT	5.30^{ad}	3.71^{bce}	1.38^{ad}	0.28^{ab}	0.31	0.69^{ab}	1.11
T17 - M AFB1 + FBs + FZYM	5.20^{abc}	3.77^{ce}	1.29^{ab}	0.26^{ab}	0.29	0.64^{ab}	1.04

Treatment	Total protein (g/L)	Albumin (g/L)	Relative liver weight	Relative spleen weight	Relative bursa weight	Relative heart weight	Relative gizzard weight
T18 - M AFB1 + FBs + BENT + FZYM	5.14[ab]	3.64[ae]	1.37[ad]	0.27[ab]	0.31	0.69[ab]	1.13
T19 – FZYM	5.06[a]	3.29[a]	1.37[ad]	0.28[ab]	0.24	0.70[ab]	1.06
T20 – BENT	5.04[a]	3.50[ac]	1.33[ac]	0.28[bc]	0.29	0.69[ab]	1.05
SEM	0.17	0.16	0.06	0.02	0.02	0.03	0.05
Main Effects				***P*-Value**			
FBs	0.0087	0.0054	NS	NS	NS	NS	NS
H AFB1	NS	NS	NS	NS	NS	NS	NS
H AFB1 + FBs	0.0437	0.0174	NS	NS	NS	0.0148	NS
M AFB1	NS	NS	NS	NS	NS	NS	NS
M AFB1 + FBs	0.0112	0.0185	NS	NS	NS	NS	NS

Treatment	Total protein (g/L)	Albumin (g/L)	Relative liver weight	Relative spleen weight	Relative bursa weight	Relative heart weight	Relative gizzard weight
FZYM	NS	NS	NS	NS	NS	NS	NS
BENT	NS	NS	NS	NS	NS	NS	NS
Interactions				***P*-Value**			
FBs vs FBs + FZYM	NS	NS	NS	NS	NS	NS	NS
FBs vs FBs + FZYM + BENT	NS	NS	NS	NS	NS	NS	NS
H AFB1 vs H AFB1 + BENT	NS	NS	NS	0.0101	NS	NS	NS
H AFB1 vs H AFB1 + BENT + FZYM	0.0361	NS	0.0055	0.0078	NS	NS	NS
H AFB1 + FBs vs H AFB1	NS	NS	NS	NS	NS	0.0115	NS
H AFB1 + FBs vs H AFB1 + FBs + BENT	NS	0.0223	NS	NS	NS	0.0195	NS

Treatment	Total protein (g/L)	Albumin (g/L)	Relative liver weight	Relative spleen weight	Relative bursa weight	Relative heart weight	Relative gizzard weight
H AFB1 + FBs vs H AFB1 + FBs + FZYM	NS	NS	NS	NS	NS	0.0409	NS
H AFB1 + FBs vs H AFB1 + FBs + BENT + FZYM	NS	NS	NS	NS	NS	0.0023	NS
M AFB1 vs M AFB1 + FBs	NS	NS	NS	NS	NS	NS	NS
M AFB1 vs M AFB1 + BENT	NS	NS	NS	NS	NS	NS	NS
M AFB1+FBs vs M AFB1 + FBs + BENT	NS	NS	NS	NS	NS	NS	NS
M AFB1 + FBs vs M AFB1 + FBs + FZYM	0.0485	NS	NS	NS	NS	NS	NS
M AFB1 + FBs vs M AFB1 + FBs + BENT + FZYM	0.0250	NS	NS	NS	NS	NS	NS

303 Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. Values within the same column not
304 sharing a common superscript differ significantly ($p < 0.05$) according to a Tukey post-hoc test. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-
305 Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite; NS-Not Significant ($p > 0.05$).

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Vaccine response

Supplementary Figure S4 shows the antibody titres against NCD vaccination at 13 d of age (one day before vaccination), 21 d of age (7 days after vaccination) and 35 d of age (21 days after vaccination) in both the control and treated groups. The dietary treatments had no significant effect on the antibody titres. Non-significantly lower antibody titres were observed in the birds fed the highest dose of AFB1, alone (T5) or in combination with FBs (T8), when compared to the control diet (T1) for all the sampling days. Serum samples collected at 21 d of age showed that birds fed both the highest dose of AFB1 and FBs (T8) had lower antibody titres compared to birds fed high AFB1 alone (T5), whereas the reverse was the case at 13 and 35 d of age, although these differences were not significant.

Aflatoxins residues in plasma, liver, and muscle tissues

Residues of AFB1 and AFM1 were detected in plasma and liver samples (Table 4). Breast muscle samples from all the experimental groups had no detectable levels of all the AFs tested (data not shown). Aflatoxin B1 concentrations were in the range of LOQ (0.05 µg/kg) to 0.12 µg/kg in liver samples. The highest AFB1 concentration was detected in liver samples of birds receiving feeds with both high AFB1 and FBs (T8), although this was not significantly different from the AFB1 residue concentration in liver of birds receiving high AFB1 only diet (T5). Inclusion of BENT in diets with high AFB1 and FBs (T9), non-significantly reduced AFB1 liver accumulation by 50% when compared to high AFB1 and FBs diets without BENT (T8). Plasma samples had detectable levels of AFB1, but below the LOQ of 0.05 ng/mL. Aflatoxin M1 was present in both plasma and liver samples of birds from high AFB1 experimental groups (T5 to T11), although at levels below the LOQs of 0.05 ng/mL and 0.1 µg/kg, respectively. Furthermore, AFM1 (<LOQ) was detected in both liver and plasma samples of birds that received diets with BENT only (T20) (Table 4). Aflatoxin G1, AFG2, AFM2 and AFB2 were not detected in plasma and liver samples from all the treatment groups.

The carry-over rates of AFB1 from feed to liver tissues are summarised in Table 4. The highest carry-over rate of 0.06% was observed in liver samples of birds that received diets with both high AFB1 and FBs (T8).

Table 4: Aflatoxin B1 (AFB1) and aflatoxin M1 (AFM1) concentrations in broiler chickens' plasma (ng/mL) and liver (µg/kg) from the different treatments at the end of the trial period (35 days), and carry-over rates of AFB1 from feed to liver (%).

Treatment	Plasma (n = 8 birds)		Liver (n = 8 birds)		
	AFB1 ± SEM (ng/mL)	AFM1 ± SEM (ng/mL)	AFB1 ± SEM (µg/kg)	Carry-over rates from feed to liver (%)	AFM1 ± SEM (µg/kg)
T1 - Control	ND	ND	ND [a]	NA	ND
T2 - FBs	<LOQ	ND	<LOQ [acd]	NA	<LOQ
T3 - FBs + FZYM	ND	ND	ND [a]	NA	ND
T4 - FBs + FZYM + BENT	<LOQ	<LOQ	<LOQ [ab]	NA	<LOQ
T5 – H AFB1	<LOQ	<LOQ	0.11 ± 0.02[e]	0.05	<LOQ
T6 - H AFB1 + BENT	<LOQ	<LOQ	0.07 ± 0.02[de]	0.03	<LOQ
T7 - H AFB1 + BENT + FZYM	ND	ND	0.09 ± 0.02[e]	0.04	<LOQ
T8 - H AFB1 + FBs	ND	ND	0.12 ± 0.03[e]	0.06	<LOQ
T9 - H AFB1 + FBs + BENT	ND	ND	0.06 ± 0.02[bce]	0.03	<LOQ
T10 - H AFB1 + FBs + FZYM	ND	ND	<LOQ [ab]	NA	ND
T11 - H AFB1 + FBs + BENT + FZYM	<LOQ	ND	0.07 ± 0.02[ce]	0.03	<LOQ
T12 - M AFB1	<LOQ	ND	<LOQ [ac]	NA	<LOQ
T13 - M AFB1 + BENT	ND	ND	<LOQ [ab]	NA	ND
T14 - M AFB1 + BENT + FZYM	<LOQ	ND	<LOQ [ab]	NA	ND

T15 - M AFB1 + FBs	ND	ND	<LOQ [ac]	NA	<LOQ
T16 - M AFB1 + FBs + BENT	<LOQ	<LOQ	<LOQ [ab]	NA	<LOQ
T17 - M AFB1 + FBs + FZYM	ND	ND	<LOQ [acd]	NA	<LOQ
T18 - M AFB1 + FBs + BENT + FZYM	ND	ND	<LOQ [ab]	NA	<LOQ
T19 – FZYM	ND	ND	ND [a]	NA	ND
T20 – BENT	ND	<LOQ	ND [a]	NA	<LOQ

Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. LOQ: limit of quantification (0.05 µg/kg for AFB1 in liver, 0.1 µg/kg for AFM1 in liver, 0.05 ng/mL for AFB1 and AFM1 in plasma,); Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) according to a Tukey post-hoc test. Carry-over rates (%) from feed into liver are expressed as a percentage of the concentration of AFB1 in the liver (µg/kg) compared to the concentration of AFB1 in the feed (µg/kg) x 100, based on 8 birds per treatment. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite; ND: Not Detected; NA: Not applicable.

Discussion

Both BENT and FZYM at the doses used in the present study (2 g BENT/kg feed and 0.012 g FZYM/kg) were safe and had no negative effect on the growth performance of the broiler chickens. This result is in agreement with other studies that reported the two detoxifiers were safe for production of broiler chickens [8,27]. The current study also showed that feeding high AFB1 only resulted in poor FCR in broilers when compared to feeding both high AFB1 and FBs, indicating that there was interaction between the two mycotoxins. Kolawole et al. [11] however observed poor FCR in broilers fed diets contaminated with multiple mycotoxins (deoxynivalenol (DON), FBs, and zearalenone (ZEN) or DON, ZEN, and diacetoxyscirpenol) below the EU guidance values. In the latter study, poor FCR was attributed to the enhanced effects due to

interactions between the mycotoxins although unlike the present study, the effects of individual mycotoxins on the FCR were not evaluated. Also presence of DON in the diets could have contributed to poor FCR since DON causes impairment of intestinal morphology including length of villus height and crypt depth, as well as length of small intestine, thus affecting absorption and utilization of nutrients [47]. The BWG of the broiler chickens was not affected by the contaminated diets and this result is consistent with previous studies in which AFB1 or FBs at levels similar or lower than levels used in the current study did not affect BWG of broiler chickens [8,15]. Tessari et al. [13] observed reduced BWG of broiler chickens fed both AFB1 (concentrations of 50 or 200 μg/kg) and FB1 (50,000 or 200,000 μg/kg). The results of the latter studies can be attributed to use of higher concentrations of dietary FBs when compared to the present study where FBs at levels of 17,430 μg/kg was used. Pappas et al. [19] also observed reduced BWG in broiler chickens fed AFB1 and OTA, both at concentrations of 100 μg/kg feed, indicating that AFB1 interacts differently with other mycotoxins. Aflatoxins cause reduced growth rates in poultry by inhibiting metabolisms and limiting protein synthesis through competition with phenylalanine for the binding sites on the phenylalanine-transfer RNA synthase [5]. Although FB1 has a low oral bioavailability of 0.7% in chickens [48], the chicken microbiota has been shown to have limited capacity to degrade FBs [8,49] and feeding chickens diets with higher FB1 at levels of 100-400 mg/kg for 2 to 3 weeks caused reduced FI and BWG [7,50].

In the present study, the highest percentage of livers with macroscopic alterations were from broilers fed diets containing high AFB1 alone or with FBs. This was an expected result and typical to subclinical aflatoxicosis and FBs toxicity as both mycotoxins target the liver. Magnoli et al.[15] reported macroscopic changes characterised by pale yellow livers in male broiler chickens fed AFB1 at levels of 50 μg/kg from 18 to 46 d of age. In the latter study, lesions consisting of hepatocellular necrosis, perilobular locations and fat vacuoles were microscopically observed in the livers of the chickens with gross pathological changes. According to Rauber et al. [10], male broiler chickens fed diets with FB1 (100 or 200 mg/kg feed) from 1 to 28 d of age had hepatocellular alterations, accompanied with lesions in the kidneys. Addition of the BENT and FZYM to contaminated diets reduced the percentage of livers with gross pathological changes, although

not all and this can be due to high AFB1 in the diets (more than 10 fold the EU legal limit of 20 μg/kg for AFB1 in poultry feeds). The high levels of AFB1 used in the current study are occasionally reported in poultry feeds from SSA[4]. In other studies, liver alterations were also observed even after addition of mycotoxin binders in contaminated diets, although a reduced magnitude and severity characterised by degeneration rather than necrosis were noted [51,52]. Neef et al.[20] observed that supplementing 0.5% HSCA to diets contaminated with AFB1 at high levels of 2,500 μg/kg did not prevent ultrastructural changes in livers of broiler chickens fed the contaminated diet from hatch to 21 d of age, indicating that at very high AFB1 levels, 0.5% HSCA failed to protect the birds.

Presence of high AFB1 and FBs in the diet increased the heart weight of the broilers in the present study. In similar findings, increased heart weights were observed in broiler chickens fed both AFB1 (50 or 200 μg/kg) and FB1 (50,000 or 200,000 μg/kg) contaminated diets from 8 to 41 d of age [13]. Pappas et al. [19] also reported increased heart weight of broiler chickens fed a diet with AFB1 and OTA, both at levels of 100 μg/kg feed. These studies show enhanced effects caused by interactions between the mycotoxins in case of co-contamination. Studies by Manna et al. [53] and Abdulmajeed [54] showed that AFB1 damaged the hearts of rats through inhibition of energy metabolism and consequent interference with energy supply in the hearts. In the present study, there was a non-significant increase in liver weights of broiler chickens fed contaminated diets. Other researchers reported that dietary AFB1 at levels of 20 to 200 μg/kg did not significantly alter liver weights [13,15], whereas higher AFB1 levels of 500 to 2,500 μg/kg feed resulted in increased broiler chickens' liver weights due to accumulation of lipids and inhibition of their transport [27,55]. The liver is considered the main target for AFB1 where bio-activation to the carcinogenic AFB1-8,9-epoxide occurs mediated by cytochrome P450 [56]. Detoxification of the AFB1-8,9-epoxide through conjugation with glutathione is also catalysed by hepatic glutathione S-transferase. The BENT at the dose used in the current study was able to reduce the negative impact of feeding AFB1 contaminated diets on the broilers heart, liver and spleen weights, demonstrating the ability of BENT to bind to AFB1 thus reducing its effects on the organs. In other findings, BENT was also reported to counteract the toxic effects

403 of AFB1 on organs of broiler chickens [19,27]. The relative gizzard and bursa weights were not different among
404 the treatment groups and in similar findings, gizzard and bursa weights of broiler chickens fed AFB1 at
405 moderate to high concentrations (20 to 500 μg/kg feed) from 1 to 35 day of age revealed no change in the
406 weights of these organs [3,57]. However, feeding AFB1 at levels higher than the present study (750 μg/kg) for
407 28 days resulted in decreased bursa weight of 7-day-old broiler chickens, confirming the impaired immune
408 functions due to aflatoxicosis [58]. As concluded for the broilers' growth performance, BENT or FZYM alone
409 at the inclusion levels in the present study were safe for use in chickens and did not affect the weights of
410 all the examined organs.

411 Increased concentrations of serum TP and ALB due to dietary FBs alone or in combination with AFB1
412 observed in the present study was similar to previous studies where dietary FBs (100,000-200,000 μg/kg
413 feed) alone or in combination with AFB1 (200 μg/kg feed) resulted in higher plasma TP and ALB . In *vitro*
414 studies by Ramasamy et al. [60] reported that FBs negatively affected endothelial cells from porcine
415 pulmonary artery and led to increased permeability of the endothelium to ALB, thereby elevating the serum
416 levels of both ALB and TP. Dietary AFB1 at low concentrations of 20 μg/kg was shown to decrease the
417 serum ALB of broiler chickens at 35 days of age [3]. Interestingly, Shannon et al. [27] reported no changes in
418 serum TP, GLB, UA and calcium of young broiler chickens that consumed dietary AFB1 up to levels of
419 2,000 μg/kg feed from hatch to day 21 of age. Blood biochemical can temporarily change depending on the
420 stage of exposure to a mycotoxin [58]. Aflatoxin B1 adducts can inhibit protein synthesis and bind to
421 hepatocytes' macromolecules, thus reducing both TP and ALB levels in the blood, resulting in
422 hypoproteinaemia [61]. Alterations in blood TP and ALB concentrations can thus constitute important
423 indicators of intoxication by mycotoxins before clinical symptoms appear. The activities of serum GGT
424 were not significantly altered by the treatments offered to broiler chickens in the current study. Blood
425 enzymes such as GGT, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate
426 aminotransferase (AST), or lactate dehydrogenase (LDH) originate mainly in the liver, but can also be
427 produced in the kidney, brain, heart, and skeletal muscle. Increased levels of these enzymes in blood were

428 associated with disruption of hepatocytes due to necrosis or altered membrane permeability during
429 aflatoxicosis [27]. Serum concentrations of CREAT, UA, and TAS were not affected by the different diets fed
430 to broiler chickens in the present study and in corresponding studies, no significant changes were observed
431 in these blood parameters when AFB1 was fed to broilers at almost similar concentrations [15,19].

432 The serum antibodies against NCD were not affected by the different diets although lower titres were
433 observed in birds fed the highest dose of AFB1 alone or in combination with FBs. Low levels of antibodies
434 against NCD or IB or IBD have been linked to immunosuppressant effects of AFB1 (200 to 500 μg/kg feed)
435 [13,58]. During aflatoxicosis, inhibition of protein synthesis can occur leading to decreased production of
436 antibodies against common viruses such as NCD and IBV, and therefore increasing susceptibility to
437 diseases [57]. Under the current experimental conditions, antibody titres against NCD were numerically lower
438 in birds fed both AFB1 and FBs when compared to groups fed the individual mycotoxins. Tessari et al. [13]
439 also reported that a combination of AFB1 and FB1 resulted in significantly lower titres against NCD as
440 compared to the individual mycotoxins. Exposure to multiple mycotoxins have been shown to cause adverse
441 effects even at low toxin levels due to interactions between the mycotoxins [11].

442 Aflatoxin B1 was detected at levels above the LOQ in livers of birds that were fed high AFB1 alone or in
443 combination with FBs and/or detoxifiers. The highest concentration of 0.12 ± 0.03 μg/kg observed in the
444 current study corresponded to a carry-over rate of 0.06%. A lower carry-over rate was reported by
445 Fernandez et al. [62] who detected mean AFB1 residues of 0.23 μg/kg in liver of 23-day-old broiler chickens
446 fed AFB1 at levels of 2,500 μg/kg for up to 32 days, corresponding to a carry-over rate of 0.009%. Hussain
447 et al. [63] reported liver AFB1 residues of 6.97 μg/kg in young broiler chickens fed dietary AFB1 at levels of
448 6,400 μg/kg for 7 days, equivalent to a carry-over rate of 0.11%. Neef et al. [20] reported higher AFB1 residues
449 of 16.16 μg/kg in liver of broiler chickens fed dietary AFB1 at levels of 2,500 μg/kg from hatch to 21 d,
450 yielding a higher carry-over rate of 0.646%. The variations in the carry-over rates observed can be attributed
451 to the different concentrations of AFB1 in the diets, age and sensitivity of the animals to AFB1 used in the
452 studies. In field studies, AFB1 was detected at mean levels of 1.7 μg/kg and 1.8 μg/kg in chicken liver and

453 muscle samples, respectively, collected from abattoirs and farms in Mozambique and Cameroon, whereas
454 in Pakistan, AFB1 levels as high as 7.86 μg/kg were detected in chickens' liver samples collected from
455 slaughter houses, shops and markets [14,16]. These studies indicate that the chickens were exposed to AFs,
456 especially through the feed. In the current study, diets with both AFB1 and FBs resulted in non-significantly
457 higher liver residual AFB1 levels when compared to feeding high AFB1 only. Pappas et al. [19] observed that
458 OTA only accumulated in tissues of broilers that received both OTA and AFB1 in their diets, indicating
459 that mycotoxins can interact with each other and enhance their accumulation in tissues, although more
460 studies should be conducted to understand the mode of interactions between different mycotoxins. Inclusion
461 of BENT in AFB1-contaminated diets non-significantly decreased the accumulation of AFB1 by up to 50%
462 in the liver of the broiler chickens in the present study. Bhatti et al. [64] reported that addition of bentonite
463 clay at levels of 3.7 or 7.5 g/kg feed in broiler chickens' diet contaminated with 600 μg AFB1/kg feed
464 resulted in over 80% reduction in liver AFB1 residues. In a study by Magnoli et al. [15], inclusion of 0.3%
465 sodium bentonite in broiler chicken diet contaminated with AFB1 at levels of 50 μg/kg feed significantly
466 reduced liver AFB1 residues. Neef et al. [20] also reported that supplementing 0.5% HSCA in male broiler
467 chickens diets with AFB1 up to levels of 2,500 μg/kg feed, reduced accumulation of AFB1 in their livers.
468 These studies demonstrate that BENT can bind to AFB1 and prevent its absorption, hence reducing it
469 transfer to tissues. At the time of the study, there were no set maximum limits for AFB1 in poultry food
470 products. However, the EU has set 2 μg/kg AFB1 as the maximum tolerance level in human food from
471 vegetal origin [65]. The highest AFB1 residue levels found in liver tissues in the present study were below
472 this tolerance level, however, regular monitoring of poultry feed and meat (especially liver) should be
473 conducted to avoid any food safety hazards.

474 Plasma samples from broilers that received the highest dose of AFB1 had detectable levels of AFB1 and
475 AFM1 but below the LOQ (0.05 ng/mL). Aflatoxin M1, which is a hydroxylated metabolite of AFB1, has
476 also been detected in tissues of animals that consumed AFB1 contaminated diets, with high levels being
477 detected in dairy animals [45]. Fernandez et al. [62] reported mean AFM1 concentrations of 0.06 μg/kg and 0.12

μg/kg in liver and kidney, respectively, of 23-day-old broiler chickens fed a diet containing AFB1 at more than 10 fold the levels used in the present study (2,500 μg/kg feed).

Breast muscle tissue from all treatment groups had no detectable levels of all AFs tested and in corresponding studies, no AFs were detected in muscle samples from broilers fed AFB1 at levels almost similar to the present study (50 to 100 μg/kg) [19,66]. However, feeding young broiler chickens very high dietary levels of AFB1 (6,400 μg/kg feed) for 7 days resulted in accumulation of AFB1 in the muscles up to concentrations of 3.27 μg/kg [63].

Considering the trace levels of AFs in liver and plasma samples in this study, poultry tissues are likely to be minor contributors to human dietary AFs intake, although frequent monitoring is required to prevent chronic exposure, especially in SSA where there is laxity to enforce regulatory limits.

Fumonisins toxicity has been linked with disruption of the sphingolipids synthesis due to their structural similarities. This disruption leads to inhibition of sphinganine (Sa) and sphingosine (So) N-acyltransferases, which are key enzymes required for the synthesis of ceramide and more complex sphingolipids [37]. Free Sa and So thus accumulate in tissues and body fluids and the increase in Sa/So ratio has been used as a biomarker for exposure and effect of FBs in broiler chickens [8,9]. The effects of feeding broiler chickens FBs alone or with FZYM on Sa/So ratio in blood and tissues were not explored in the current study and warrant evaluation in future *in vivo* broiler trials aiming at studying FBs toxicity or potential mycotoxin detoxifiers for counteracting FBs.

In conclusion, feeding AFB1 at levels of 220 or 60 μg/kg feed, and FBs at a level of 17,430 μg/kg feed, alone or in combination, had no negative impact on the growth of the broiler chickens. However, high AFB1 alone (220 μg/kg feed) or in combination with FBs (17,430 μg/kg feed), caused changes in FCR and heart weights of the broiler chickens. Serum total protein and albumin concentrations were altered by feeding FBs only (17,430 μg/kg feed) or in combination with high (220 μg/kg feed) or moderate AFB1 (60 μg/kg feed) or the detoxifiers. Low carry-over rates of AFB1 from feed to liver tissues were observed, with

the maximum concentration of 0.12 ± 0.03 μg/kg being detected in livers of chickens that received a diet with both high AFB1 (220 μg/kg feed) and FBs (17,430 μg/kg feed). Furthermore, BENT and FZYM at the doses tested were safe and efficient to counteract some of the negative effects of AFB1 and FBs, respectively. This study therefore provides more evidence on the effects of AFB1 and FBs on health and productivity of broiler chickens and use of mycotoxin detoxifiers as a sustainable post-harvest mitigation strategy to counteract these effects, especially in SSA where reliable mycotoxin testing of feeds is not frequent.

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ASSOCIATED CONTENT

Supporting Information

Supplementary Figure S1 and S2 show chemical structures of major aflatoxins and fumonisins reported in food and feed as well as the structure of partially hydrolysed fumonisin B1a and B1b and fully hydrolysed fumonisin B1. Figure S3 shows the percent of liver samples with gross pathological changes while figure

S4 shows antibody titres against NCD for the control and treated groups. Table S1 shows the chemical and mycotoxin composition of control diet and the EU regulatory/guidance values for the major mycotoxins (PDF).

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References

(1) Kemboi, D. C.; Ochieng, P. E.; Antonissen, G.; Croubels, S.; Scippo, M.-L.; Okoth, S.; Kangethe, E. K.; Faas, J.; Doupovec, B.; Lindahl, J. F. et al. Multi-Mycotoxin Occurrence in Dairy Cattle and Poultry Feeds and Feed Ingredients from Machakos Town, Kenya. *Toxins* **2020**, *12* (12), 762. https://doi.org/10.3390/toxins12120762.

(2) Dooso Oloo, R.; Okoth, S.; Wachira, P.; Mutiga, S.; Ochieng, P.; Kago, L.; Nganga, F.; Domelevo Entfellner, J.-B.; Ghimire, S. Genetic Profiling of Aspergillus Isolates with Varying Aflatoxin Production Potential from Different Maize-Growing Regions of Kenya. *Toxins* **2019**, *11* (8), 467. https://doi.org/10.3390/toxins11080467.

(3) Saminathan, M.; Selamat, J.; Abbasi Pirouz, A.; Abdullah, N.; Zulkifli, I. Effects of Nano-Composite Adsorbents on the Growth Performance, Serum Biochemistry, and Organ Weights of Broilers Fed with Aflatoxin-Contaminated Feed. *Toxins* **2018**, *10* (9), 345. https://doi.org/10.3390/toxins10090345.

(4) Ochieng, P. E.; Scippo, M.-L.; Kemboi, D. C.; Croubels, S.; Okoth, S.; Kang'ethe, E. K.; Doupovec, B.; Gathumbi, J. K.; Lindahl, J. F.; Antonissen, G. Mycotoxins in Poultry Feed and Feed Ingredients from Sub-Saharan Africa and Their Impact on the Production of Broiler and Layer Chickens: A Review. *Toxins* **2021**, *13* (9), 633. https://doi.org/10.3390/toxins13090633.

(5) Monson, M.; Coulombe, R.; Reed, K. Aflatoxicosis: Lessons from Toxicity and Responses to Aflatoxin B1 in Poultry. *Agriculture* **2015**, *5* (3), 742–777. https://doi.org/10.3390/agriculture5030742.

(6) Sewram, V.; Mshicileli, N.; Shephard, G. S.; Vismer, H. F.; Rheeder, J. P.; Lee, Y.-W.; Leslie, J. F.; Marasas, W. F. O. Production of Fumonisin B and C Analogues by Several *Fusarium* Species. *J. Agric. Food Chem.* **2005**, *53* (12), 4861–4866. https://doi.org/10.1021/jf050307n.

(7) Javed, T.; Bennett, G. A.; Richard, J. L.; Dombrink-Kurtzman, M. A.; Côté, L. M.; Buck, W. B. Mortality in Broiler Chicks on Feed Amended WithFusarium Proliferatum Culture Material or with Purified Fumonisin B1 and Moniliformin. *Mycopathologia* **1993**, *123* (3), 171–184. https://doi.org/10.1007/BF01111269.

(8) Grenier, B.; Schwartz-Zimmermann, H. E.; Gruber-Dorninger, C.; Dohnal, I.; Aleschko, M.; Schatzmayr, G.; Moll, W. D.; Applegate, T. J. Enzymatic Hydrolysis of Fumonisins in the Gastrointestinal Tract of Broiler Chickens. *Poult. Sci.* **2017**, *96* (12), 4342–4351. https://doi.org/10.3382/ps/pex280.

(9) Antonissen, G.; Croubels, S.; Pasmans, F.; Ducatelle, R.; Eeckhaut, V.; Devreese, M.; Verlinden, M.; Haesebrouck, F.; Eeckhout, M.; De Saeger, S. et al. Fumonisins Affect the Intestinal Microbial Homeostasis in Broiler Chickens, Predisposing to Necrotic Enteritis. *Vet. Res.* **2015**, *46* (1). https://doi.org/10.1186/s13567-015-0234-8.

(10) Rauber, R. H.; Dilkin, P.; Mallmann, A. O.; Marchioro, A.; Mallmann, C. A.; Borsoi, A.; Nascimento, V. P. Individual and Combined Effects of Salmonella Typhimurium Lipopolysaccharide and Fumonisin B1 in Broiler Chickens. *Poult. Sci.* **2012**, *91* (11), 2785–2791. https://doi.org/10.3382/ps.2012-02489.
(11) Kolawole, O.; Graham, A.; Donaldson, C.; Owens, B.; Abia, W. A.; Meneely, J.; Alcorn, M. J.; Connolly, L.; Elliott, C. T. Low Doses of Mycotoxin Mixtures below EU Regulatory Limits Can Negatively Affect the Performance of Broiler Chickens: A Longitudinal Study. *Toxins* **2020**, *12* (7), 433. https://doi.org/10.3390/toxins12070433.
(12) Njobeh, P. B.; Dutton, M. F.; Åberg, A. T.; Haggblom, P. Estimation of Multi-Mycotoxin Contamination in South African Compound Feeds. *Toxins* **2012**, *4* (10), 836–848. https://doi.org/10.3390/toxins4100836.
(13) Tessari, E. N. C.; Oliveira, C. A. F.; Cardoso, A. L. S. P.; Ledoux, D. R.; Rottinghaus, G. E. Effects of Aflatoxin B_1 and Fumonisin B_1 on Body Weight, Antibody Titres and Histology of Broiler Chicks. *Br. Poult. Sci.* **2006**, *47* (3), 357–364. https://doi.org/10.1080/00071660600756071.
(14) Sineque, A.; Macuamule, C.; Dos Anjos, F. Aflatoxin B1 Contamination in Chicken Livers and Gizzards from Industrial and Small Abattoirs, Measured by ELISA Technique in Maputo, Mozambique. *Int. J. Environ. Res. Public. Health* **2017**, *14* (9), 951. https://doi.org/10.3390/ijerph14090951.
(15) Magnoli, A. P.; Monge, M. P.; Miazzo, R. D.; Cavaglieri, L. R.; Magnoli, C. E.; Merkis, C. I.; Cristofolini, A. L.; Dalcero, A. M.; Chiacchiera, S. M. Effect of Low Levels of Aflatoxin B1 on Performance, Biochemical Parameters, and Aflatoxin B1 in Broiler Liver Tissues in the Presence of Monensin and Sodium Bentonite. *Poult. Sci.* **2011**, *90* (1), 48–58. https://doi.org/10.3382/ps.2010-00971.
(16) Iqbal, S. Z.; Nisar, S.; Asi, M. R.; Jinap, S. Natural Incidence of Aflatoxins, Ochratoxin A and Zearalenone in Chicken Meat and Eggs. *Food Control* **2014**, *43*, 98–103. https://doi.org/10.1016/j.foodcont.2014.02.046.
(17) Laurain, J.; Tardieu, D.; Matard-Mann, M.; Rodriguez, M. A.; Guerre, P. Fumonisin B1 Accumulates in Chicken Tissues over Time and This Accumulation Was Reduced by Feeding Algo-Clay. *Toxins* **2021**, *13* (10), 701. https://doi.org/10.3390/toxins13100701.
(18) Tardieu, D.; Travel, A.; Le Bourhis, C.; Metayer, J.-P.; Mika, A.; Cleva, D.; Boissieu, C.; Guerre, P. Fumonisins and Zearalenone Fed at Low Levels Can Persist Several Days in the Liver of Turkeys and Broiler Chickens after Exposure to the Contaminated Diet Was Stopped. *Food Chem. Toxicol.* **2021**, *148*, 111968. https://doi.org/10.1016/j.fct.2021.111968.
(19) Pappas, A. C.; Tsiplakou, E.; Tsitsigiannis, D. I.; Georgiadou, M.; Iliadi, M. K.; Sotirakoglou, K.; Zervas, G. The Role of Bentonite Binders in Single or Concomitant Mycotoxin Contamination of Chicken Diets. *Br. Poult. Sci.* **2016**, *57* (4), 551–558. https://doi.org/10.1080/00071668.2016.1187712.
(20) Neeff, D. V.; Ledoux, D. R.; Rottinghaus, G. E.; Bermudez, A. J.; Dakovic, A.; Murarolli, R. A.; Oliveira, C. A. F. In Vitro and in Vivo Efficacy of a Hydrated Sodium Calcium Aluminosilicate to Bind and Reduce Aflatoxin Residues in Tissues of Broiler Chicks Fed Aflatoxin B1. *Poult. Sci.* **2013**, *92* (1), 131–137. https://doi.org/10.3382/ps.2012-02510.
(21) Vila-Donat, P.; Marín, S.; Sanchis, V.; Ramos, A. J. A Review of the Mycotoxin Adsorbing Agents, with an Emphasis on Their Multi-Binding Capacity, for Animal Feed

Decontamination. *Food Chem. Toxicol.* **2018**, *114*, 246–259. https://doi.org/10.1016/j.fct.2018.02.044.
(22) Heinl, S.; Hartinger, D.; Thamhesl, M.; Vekiru, E.; Krska, R.; Schatzmayr, G.; Moll, W.-D.; Grabherr, R. Degradation of Fumonisin B1 by the Consecutive Action of Two Bacterial Enzymes. *J. Biotechnol.* **2010**, *145* (2), 120–129. https://doi.org/10.1016/j.jbiotec.2009.11.004.
(23) EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Rychen, G.; Aquilina, G.; Azimonti, G.; Bampidis, V.; de Lourdes Bastos, M.; Bories, G.; Chesson, A.; Cocconcelli, P. S.; Flachowsky, G.; Gropp, J.et al. Safety and Efficacy of Fumonisin Esterase (FUMzyme®) as a Technological Feed Additive for All Avian Species. *EFSA J.* **2016**, *14* (11). https://doi.org/10.2903/j.efsa.2016.4617.
(24) EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Scientific Opinion on the Safety and Efficacy of Bentonite as a Technological Feed Additive for All Species. *EFSA J.* **2012**, *10* (7). https://doi.org/10.2903/j.efsa.2012.2787.
(25) European Commission. Regulation (European Commission) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition. *Off. J. Eur. Union*, **2003**, *L 268*, 29–43.
(26) European Register: Food Safety. Feed Additives. Available Online: Https://Food.Ec.Europa.Eu/Safety/Animal-Feed/Feed-Additives/Eu-Register_en/ (Accessed on 15 December 2022).
(27) Shannon, T. A.; Ledoux, D. R.; Rottinghaus, G. E.; Shaw, D. P.; Daković, A.; Marković, M. The Efficacy of Raw and Concentrated Bentonite Clay in Reducing the Toxic Effects of Aflatoxin in Broiler Chicks. *Poult. Sci.* **2017**, *96* (6), 1651–1658. https://doi.org/10.3382/ps/pew408.
(28) Ochieng, P. E.; Kemboi, D. C.; Scippo, M.-L.; Gathumbi, J. K.; Kangethe, E.; Doupovec, B.; Croubels, S.; Lindahl, J. F.; Antonissen, G.; Okoth, S. Maximizing Laboratory Production of Aflatoxins and Fumonisins for Use in Experimental Animal Feeds. *Microorganism* **2022**, *10* (12), 2385. https://doi.org/10.3390/microorganisms10122385.
(29) Amakhobe, T. V.; Okoth, S.; Wachira, P. Mycoflora, Aflatoxin, and Fumonisin Levels Present in Layer Feed in Nairobi County. *Afr. J. Biol. Sci.* **2021**, *3* (4), 20. https://doi.org/10.33472/AFJBS.3.4.2021.20-32.
(30) Okoth, S.; De Boevre, M.; Vidal, A.; Diana Di Mavungu, J.; Landschoot, S.; Kyallo, M.; Njuguna, J.; Harvey, J.; De Saeger, S. Genetic and Toxigenic Variability within Aspergillus Flavus Population Isolated from Maize in Two Diverse Environments in Kenya. *Front. Microbiol.* **2018**, *9*. https://doi.org/10.3389/fmicb.2018.00057.
(31) Monbaliu, S.; Van Poucke, C.; Detavernier, C.; Dumoulin, F.; Van De Velde, M.; Schoeters, E.; Van Dyck, S.; Averkieva, O.; Van Peteghem, C.; De Saeger, S. Occurrence of Mycotoxins in Feed as Analyzed by a Multi-Mycotoxin LC-MS/MS Method. *J. Agric. Food Chem.* **2010**, *58* (1), 66–71. https://doi.org/10.1021/jf903859z.
(32) *Nutrient Requirements of Poultry: Ninth Revised Edition, 1994*; National Academies Press: Washington, D.C., 1994; p 2114. https://doi.org/10.17226/2114.
(33) Sulyok, M.; Stadler, D.; Steiner, D.; Krska, R. Validation of an LC-MS/MS-Based Dilute-and-Shoot Approach for the Quantification of > 500 Mycotoxins and Other Secondary Metabolites in Food Crops: Challenges and Solutions. *Anal. Bioanal. Chem.* **2020**, *412* (11), 2607–2620. https://doi.org/10.1007/s00216-020-02489-9.

(34) European Commission. Commission Recommendation of 17 August 2006 on the Prevention and Reduction of Fusarium Toxins in Cereals and Cereal Products. *Off. J. Eur. Union*, **2006**, *L 234*, 35–40.
(35) European Commission. Commission Recommendation 2006/576/EC of 17 August 2006 on the Presence of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 and HT-2 Toxin and Fumonisins in Products Intended for Animal Feeding. *Off. J. Eur. Union*, **2006**, *L 229*, 7–9.
(36) European Commission. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on Undesirable Substances in Animal Feed - Council Statement. *Off. J. Eur. Union*, **2002**, *L 140*, 10–22.
(37) Antonissen, G.; De Baere, S.; Novak, B.; Schatzmayr, D.; den Hollander, D.; Devreese, M.; Croubels, S. Toxicokinetics of Hydrolyzed Fumonisin B1 after Single Oral or Intravenous Bolus to Broiler Chickens Fed a Control or a Fumonisins-Contaminated Diet. *Toxins* **2020**, *12* (6), 413. https://doi.org/10.3390/toxins12060413.
(38) Awad, W. A.; Bohm, J.; Razzazi-Fazeli, E.; Zentek, J. Effects of Feeding Deoxynivalenol Contaminated Wheat on Growth Performance, Organ Weights and Histological Parameters of the Intestine of Broiler Chickens*. *J. Anim. Physiol. Anim. Nutr.* **2006**, *90* (1–2), 32–37. https://doi.org/10.1111/j.1439-0396.2005.00616.x.
(39) Gough, R. E.; Allan, W. H. Aerosol Vaccination against Newcastle Disease Using the Ulster Strain. *Avian Pathol.* **1976**, *5* (2), 81–95. https://doi.org/10.1080/03079457608418174.
(40) Sakamoto, M. I.; Murakami, A. E.; Fernandes, A. M.; Ospina-Rojas, I. C.; Nunes, K. C.; Hirata, A. K. Performance and Serum Biochemical Profile of Japanese Quail Supplemented with Silymarin and Contaminated with Aflatoxin B1. *Poult. Sci.* **2018**, *97* (1), 159–166. https://doi.org/10.3382/ps/pex277.
(41) De Baere, S.; Ochieng, P. E.; Kemboi, D. C.; Scippo, M.-L.; Okoth, S.; Lindahl, J. F.; Gathumbi, J. K.; Antonissen, G.; Croubels, S. Development of High-Throughput Sample Preparation Procedures for the Quantitative Determination of Aflatoxins in Biological Matrices of Chickens and Cattle Using UHPLC-MS/MS. *Toxins* **2023**, *15* (1), 37. https://doi.org/10.3390/toxins15010037.
(42) European Commission. Commission Decision 2002/657/EC, Aug 12, 2002, Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results. *Off. J. Eur. Communities* **2002**, No. L221, 8–36.
(43) R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from Https://Www. r-Project.Org/. **2020**.
(44) Swamy, H.; Smith, T.; Cotter, P.; Boermans, H.; Sefton, A. Effects of Feeding Blends of Grains Naturally Contaminated with Fusarium Mycotoxins on Production and Metabolism in Broilers. *Poult. Sci.* **2002**, *81* (7), 966–975. https://doi.org/10.1093/ps/81.7.966.
(45) Kemboi, D.; Antonissen, G.; Ochieng, P.; Croubels, S.; De Baere, S.; Scippo, M.-L.; Okoth, S.; Kangethe, E.; Faas, J.; Doupovec, B. et al. Efficacy of Bentonite and Fumonisin Esterase in Mitigating the Effects of Aflatoxins and Fumonisins in Two Kenyan Cattle Breeds. *J. Agric. Food Chem.* **2023**. https://doi.org/10.1021/acs.jafc.2c08217.
(46) Meerpoel, C.; Vidal, A.; Tangni, E. K.; Huybrechts, B.; Couck, L.; De Rycke, R.; De Bels, L.; De Saeger, S.; Van den Broeck, W.; Devreese, M. et al. A Study of Carry-Over and Histopathological Effects after Chronic Dietary Intake of Citrinin in Pigs, Broiler Chickens and Laying Hens. *Toxins* **2020**, *12* (11), 719. https://doi.org/10.3390/toxins12110719.

(47) Riahi, I.; Marquis, V.; Ramos, A. J.; Brufau, J.; Esteve-Garcia, E.; Pérez-Vendrell, A. M. Effects of Deoxynivalenol-Contaminated Diets on Productive, Morphological, and Physiological Indicators in Broiler Chickens. *Animals* **2020**, *10* (10), 1795. https://doi.org/10.3390/ani10101795.
(48) Vudathala, K. D.; Prelusky B., Dr. D.; Mejid Ayroud; Trenholm, J. D. M. Pharmacokinetic Fate and Pathological Effects of 14C-Fumonisin B1 in Laying Hens. *Natural Toxins* **1994**, *2* (2), 81–88.
(49) Masching, S.; Naehrer, K.; Schwartz-Zimmermann, H.-E.; Sărăndan, M.; Schaumberger, S.; Dohnal, I.; Nagl, V.; Schatzmayr, D. Gastrointestinal Degradation of Fumonisin B1 by Carboxylesterase FumD Prevents Fumonisin Induced Alteration of Sphingolipid Metabolism in Turkey and Swine. *Toxins* **2016**, *8* (3), 84. https://doi.org/10.3390/toxins8030084.
(50) Ledoux, D. R.; Brown, T. P.; Weibking, T. S.; Rottinghaus, G. E. Fumonisin Toxicity in Broiler Chicks. *J. Vet. Diagn. Invest.* **1992**, *4* (3), 330–333. https://doi.org/10.1177/104063879200400317.
(51) Stefanović, D.; Marinković, D.; Trailović, S.; Vasiljević, M.; Farkaš, H.; Raj, J.; Tolimir, N.; Radulović, S.; Nešić, V.; Trailović, J. N. et al. Evaluation of Effectiveness of a Novel Multicomponent Mycotoxins Detoxification Agent in the Presence of AFB1 and T-2 Toxin on Broiler Chicks. *Microorganisms* **2023**, *11* (3), 574. https://doi.org/10.3390/microorganisms11030574.
(52) Zabiulla, I.; Malathi, V.; Swamy, H. V. L. N.; Naik, J.; Pineda, L.; Han, Y. The Efficacy of a Smectite-Based Mycotoxin Binder in Reducing Aflatoxin B1 Toxicity on Performance, Health and Histopathology of Broiler Chickens. *Toxins* **2021**, *13* (12), 856. https://doi.org/10.3390/toxins13120856.
(53) Mannaa, F. A.; Abdel-Wahhab, K. G.; Abdel-Wahhab, M. A. Prevention of Cardiotoxicity of Aflatoxin B1 via Dietary Supplementation of Papaya Fruit Extracts in Rats. *Cytotechnology* **2014**, *66* (2), 327–334. https://doi.org/10.1007/s10616-013-9579-x.
(54) Ameen Abdulmajeed, N. Therapeutic Ability of Some Plant Extracts on Aflatoxin B1 Induced Renal and Cardiac Damage. *Arab. J. Chem.* **2011**, *4* (1), 1–10. https://doi.org/10.1016/j.arabjc.2010.06.005.
(55) Aikore, M. O. S.; Ortega-Beltran, A.; Eruvbetine, D.; Atehnkeng, J.; Falade, T. D. O.; Cotty, P. J.; Bandyopadhyay, R. Performance of Broilers Fed with Maize Colonized by Either Toxigenic or Atoxigenic Strains of Aspergillus Flavus with and without an Aflatoxin-Sequestering Agent. *Toxins* **2019**, *11* (10), 565. https://doi.org/10.3390/toxins11100565.
(56) Hsieh, D. P.; Lin, M. T.; Yao, R. C.; Singh, R. Biosynthesis of Aflatoxin. Conversion of Norsolorinic Acid and Other Hypothetical Intermediates into Aflatoxin B1. *J. Agric. Food Chem.* **1976**, *24* (6), 1170–1174.
(57) Mesgar, A.; Shahryar, H. A.; Bailey, C. A.; Ebrahimnezhad, Y.; Mohan, A. Effect of Dietary L-Threonine and Toxin Binder on Performance, Blood Parameters, and Immune Response of Broilers Exposed to Aflatoxin B1. **2022**, 23.
(58) Yunus, A. W.; Böhm, J. Temporary Modulation of Responses to Common Vaccines and Serum Cation Status in Broilers during Exposure to Low Doses of Aflatoxin B1. *Poult. Sci.* **2013**, *92* (11), 2899–2903. https://doi.org/10.3382/ps.2013-03363.
(59) Tessari, E. N. C.; Kobashigawa, E.; Cardoso, A. L. S. P.; Ledoux, D. R.; Rottinghaus, G. E.; Oliveira, C. A. F. Effects of Aflatoxin B1 and Fumonisin B1 on Blood Biochemical

Parameters in Broilers. *Toxins* **2010**, *2* (4), 453–460. https://doi.org/10.3390/toxins2040453.
(60) Ramasamy, S.; Wang; Hennig, B.; Merrill. Fumonisin B1 Alters Sphingolipid Metabolism and Disrupts the Barrier Function of Endothelial Cells in Culture. *Toxicology and applied pharmacology* **1995**, *133* (2), 343–348.
(61) Tung, H.-T.; Cook, F. W.; Wyatt, R. D.; Hamilton, P. B. The Anemia Caused by Aflatoxin. *Poult. Sci.* **1975**, *54* (6), 1962–1969. https://doi.org/10.3382/ps.0541962.
(62) Fernandez, A.; Verde, M. T.; Gascon, M.; Ramos, J.; Gomez, J.; Luco, D. F.; Chavez, G. Variations of Clinical Biochemical Parameters of Laying Hens and Broiler Chickens Fed Aflatoxin-containing Feed. *Avian Pathol.* **1994**, *23* (1), 37–47. https://doi.org/10.1080/03079459408418973.
(63) Hussain, Z.; Khan, M. Z.; Khan, A.; Javed, I.; Saleemi, M. K.; Mahmood, S.; Asi, M. R. Residues of Aflatoxin B1 in Broiler Meat: Effect of Age and Dietary Aflatoxin B1 Levels. *Food Chem. Toxicol.* **2010**, *48* (12), 3304–3307. https://doi.org/10.1016/j.fct.2010.08.016.
(64) Bhatti, S. A.; Khan, M. Z.; Hassan, Z. U.; Saleemi, M. K.; Saqib, M.; Khatoon, A.; Akhter, M. Comparative Efficacy of Bentonite Clay, Activated Charcoal and *Trichosporon Mycotoxinivorans* in Regulating the Feed-to-Tissue Transfer of Mycotoxins: Regulating Mycotoxins Deposition in Liver. *J. Sci. Food Agric.* **2018**, *98* (3), 884–890. https://doi.org/10.1002/jsfa.8533.
(65) European Commission. Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Off. J. Eur. Union*, **2006**, *L 364*, 5–24.
(66) Hussain, Z.; Rehman, H.-; Manzoor, S.; Tahir, S.; Mukhtar, M. Determination of Liver and Muscle Aflatoxin B1 Residues and Select Serum Chemistry Variables during Chronic Aflatoxicosis in Broiler Chickens. *Vet. Clin. Pathol.* **2016**, *45* (2), 330–334. https://doi.org/10.1111/vcp.12336.

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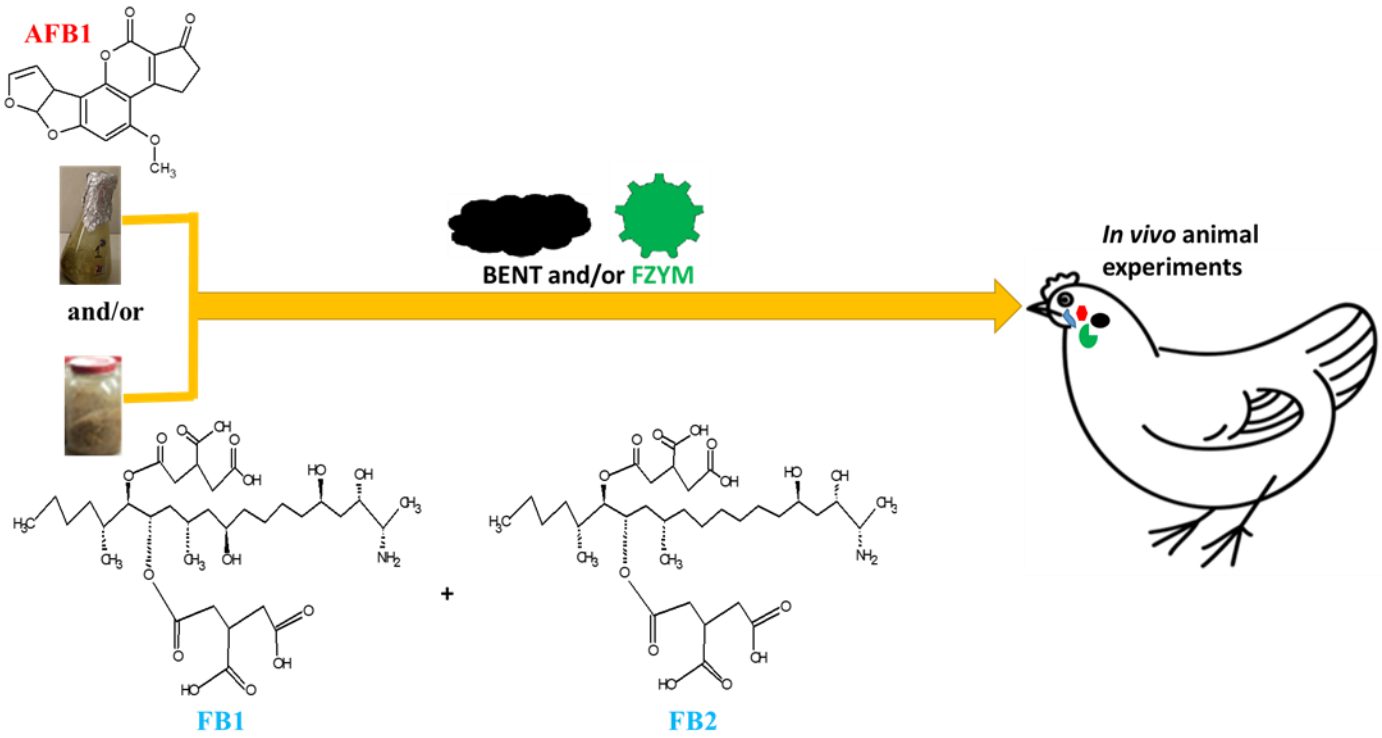

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778 **Figure 1:** Graphical abstract of in vivo trials that involved feeding broiler chickens diets with AFB1 or

779 FBs (FB1+FB2), or both and in selected diets BENT and/or FZYM were added. FBs-Fumonisins; AFB1-

780 Aflatoxin B1; FZYM-Fumonisin esterase; BENT-Bentonite.

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