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Efficacy of Bentonite and Fumonisin Esterase in Mitigating the Effects of Aflatoxins and Fumonisins in Two Kenyan Cattle Breeds direri@chuka.ac.ke

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ABSTRACT: The objective of the study was to investigate the efficacy of bentonite and fumonisin esterase, separately or combined, in mitigating the effects of aflatoxins (AF) and fumonisins (FUM) in Boran and Friesian-Boran crossbreed cattle. These effects were studied by measuring mycotoxins, their metabolites, and biomarkers that relate to animal health, productivity, and food safety. The study was divided into three experiments each lasting for 2 weeks. Cows in experiment 1 received in random order aflatoxin B1 (AFB1) [788 μg/cow/day (69.7 μg/kg dry matter intake (DMI)) for Borans and 2,310 μg/cow/day (154 μg/kg DMI) for crossbreeds], bentonite (60 g/cow/day), or both AFB1 and bentonite. Boran cows in experiment 2 received in random order FUM (12.4 mg/cow/day (1.1 mg/kg DMI)), fumonisin esterase (120 U/cow/day), or both FUM and fumonisin esterase. Boran cows in experiment 3 received in random order AFB1 (952 μg/cow/day (84.2 μg/kg DMI)) + FUM (30.4 mg/cow/day (2.7 mg/kg DMI)), bentonite (60 g/cow/day) + fumonisin esterase (120 U/cow/day), or both AFB1 + FUM and bentonite + fumonisin esterase. Feeding AFB1 and/or FUM contaminated feed with or without the addition of the detoxifiers for 14 days did not affect DMI, milk composition, hematology, and blood biochemical parameters. The addition of bentonite in a diet contaminated with AFB1 led to a decrease in milk aflatoxin M1 (AFM1) concentration of 30% and 43%, with the carry-over subsequently decreasing from 0.35% to 0.20% and 0.08% to 0.06% for crosses and Borans, respectively. No significant change was observed in the sphinganine/sphingosine (Sa/So) ratio following feeding with FUM alone or in combination with fumonisin esterase; however, the ability of fumonisin esterase to hydrolyze FUM into less toxic fully hydrolyzed FUM and partially hydrolyzed FUM was evident in the rumen fluid and feces. These results indicate bentonite was effective in decreasing AFM1 concentration in milk, and AFB1 and AFM1 in plasma, while fumonisin esterase can convert FUM into less toxic metabolites and can be a suitable addition to feed cocontaminated with AFB1 and FUM.

KEYWORDS: *adsorbent, aflatoxins, aflatoxin M1 carry-over, bentonite, biomarkers, cocontamination, fumonisin esterase, fumonisins, Sa/So ratio*

1. INTRODUCTION

Mycotoxins are low molecular weight secondary metabolites that are common contaminants of cereal crops, more commonly maize, wheat, cotton, sunflower, and peanuts.[1] These crops and their byproducts are used as ingredients in animal feeds making them also prone to contamination.[2] *Aspergillus* and *Fusarium* are the major mycotoxin-producing fungi of agricultural importance and cause contamination in the field, during harvesting, transport, or at storage.[3] Of the mycotoxins, aflatoxins (AF) and fumonisins (FUM) are the most studied and frequently occurring mycotoxins in dairy feed and feed ingredients in sub-Saharan Africa (SSA) and in most instances co-occur.[4]

Following their consumption, both AF and FUM pose animal health and productivity concerns. Aflatoxin B1 (AFB1) causes hepatotoxicity, nephrotoxicity, and immunosuppression and is further classified as a class 1 carcinogen in humans.[5] Once absorbed, AF or their metabolites can be detected in plasma and are useful biomarkers of exposure.[6,7] Ruminants are more tolerant to FUM, but at the exposure of high doses over a longer time, effects such as reduced feed intake, decrease in milk yield, hepatotoxicity, nephrotoxicity, and reproduction effects have been reported.[8−11] Structurally, FUM are similar to sphingosine (So) and sphinganine (Sa) and interfere with sphingoid bases metabolism by blocking ceramide synthase causing an increase in intercellular free Sa.[12] Therefore, this variation of Sa/So ratio characterized by elevated Sa in blood and urine samples may be used as a biomarker of exposure and effect of FUM.[13−16] The liver is the main target organ for both AF and FUM, resulting in impairment of protein synthesis leading to reduced levels of plasma proteins and amino acids,[17] and causing anemia, lymphocytopenia, and monocytopenia.[18−21] AF further affects the liver with plasma glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase

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(GPT), γ-glutamyl transferase (GGT), and alkaline phosphatase (ALP) used as biochemical indicators.[18,19,22] Long-term low-level exposure to FUM elevates serum aspartate aminotransferase (AST) and GGT.[23] However, little has been done to show the impact of FUM on hematological and biochemical parameters in dairy cattle. Additionally, most of these studies in dairy cattle have focused on the individual effects of AF and FUM. Therefore, there is a need to understand the impact of their co-occurrence in dairy cattle.

Aflatoxin M1 (AFM1) is the monohydroxylated metabolite of AFB1 which is similarly toxic and carcinogenic and is secreted in the milk of cows fed on AFB1-contaminated diets.[5,24] Analysis of AFM1 in milk is therefore of importance in checking for food safety and AFB1 exposure, using several methods including chromatography. This carry-over of AFB1 to milk as AFM1 ranges from less than 1% to 6.2% and may be affected by, among other factors, the level of milk production, breed, and presence of other mycotoxins.[24−27] Fumonisins on the other hand are not excreted in milk but have a synergistic effect when they co-occur with AF in pigs[13,28] and humans in the induction of liver disease.[29] Hence, we hypothesize that the co-occurrence of AF and FUM may increase the uptake of AF and its subsequent carry-over to milk.

Decontamination is one of the measures used in the livestock sector to mitigate the effects of mycotoxins. Detoxifiers are applied to contaminated feed to reduce the bioavailability of the toxin.[8] The use of such detoxifiers is practical and effective with studies on the use of binders such as bentonite at an inclusion rate of up to 2% showing up to 60% reduction in AFM1 carry-over in cattle fed AFB1 contaminated feed.[22,30−32] However, binders are not effective against FUM with modifiers being the better alternative. Fumonisin esterase (FUM*zyme* by Biomin GmbH, part of DSM) is an enzyme produced by *Komagataella phaffii* DSM 32159. It cleaves FUM resulting in the formation of fully hydrolyzed FUM and partially hydrolyzed FUM and is effective in poultry and pigs.[33,34] However, little has been done to check the effectiveness of bentonite and fumonisin esterase in the case of co-occurrence of AF and FUM in the feed of dairy cattle.

This study aimed at evaluating the efficacy of bentonite and fumonisin esterase, separately or combined, in mitigating the effects of AF and FUM in Boran and Friesian-Boran crossbreed cattle. More specifically, AFM1 carry-over to milk, plasma hematological, and biochemical parameters, dry matter intake (DMI), and milk production and composition were studied.

2. MATERIAL AND METHODS

2.1. Preparation of Mycotoxins and Detoxifiers. The mycotoxins, AFB1 and FUM, used in the study were produced at the Mycology and Mycotoxin Laboratory, University of Nairobi, Kenya, by cultivating known AF-producing fungal strains of *A. flavus* (17S, 121365S, and 86S) and FUM-producing fungal strains of *F. verticillioides* (K52, K826, and K81C). The procedure used was as described by Ochieng et al.[35] which was based on the method by Okoth et al.[36] with minor modifications. Maize was used as the carrier, and moisture was adjusted by soaking in sterile distilled water at room temperature overnight before being sterilized by autoclaving (121 °C, 20 min). For FUM production, the maize was first cracked before soaking. The maize was inoculated with the three *A. flavus* strains and incubated at 29 °C under darkness for 3 weeks for AF production. For FUM production, the maize was inoculated with the three strains of *F. verticillioides*, and incubated at 22−25 °C in a chamber fitted with yellow light for 3 weeks. Then, the inoculated carrier was oven-dried for 2 days before being separately milled to a fine powder using a blender. Culture materials were analyzed by an HPLC method at the International Livestock Research Institute (ILRI), Nairobi, Kenya, for AF and Kenya Plant Health Inspectorate (KEPHIS) for FUM.

FUM*zyme* (FZYM) and Mycofix Secure (MFX) both from Biomin GmbH (Getzersdorf, Austria, part of DSM) were used as mycotoxin detoxifiers. FUMzyme acts by cleaving the tricarballylic acid side chains of FUM, resulting in the formation of nontoxic hydrolyzed fumonisins (HFBs) in the gastrointestinal tract. Mycofix Secure is a mycotoxin binder composed of bentonite (dioctahedral montmorillonite), designed to counter the effects of AF in animal feed.[37] Both detoxifiers have been approved for use in dairy cattle and are administered by mixing in concentrate feed within the manufacturer's recommended levels of 60 g/cow/day for Mycofix Secure and 120 Units/cow/day for FUM*zyme*.

2.2. Animal Selection, Care, and Housing. The experiment was conducted at the ILRI, Nairobi, Kenya. All the experimental procedures and protocols were approved by the ILRI's Institutional Animal Care and Use Committee (approval IACUC RC-2019-04).

Cows were housed in stalls with 2 animals per stall and with sawdust bedding, and *ad libitum* hay and water access. The animals were accompanied by their calves to ensure continuous milk production during the period of the trial.

Twenty-four animals (18 Borans and 6 crosses) in the early lactation (days in milk (mean ± SD) = 30.7 ± 5.7, body weight (mean ± SD) = 341.0 ± 33.8 kg) were used. The average DMI (mean ± SD) for the Borans and crosses was 11.3 ± 1.3 and 15.0 ± 1.4 kg, respectively. The Borans were randomly divided into 3 groups of 6 animals each and assigned to one of the 3 experiments, while the 6 crosses were only used for experiment 1. The inclusion of the crosses in experiment 1 only was due to the limitation on the availability of animals at early lactation to be used for the other experiments.

2.3. Experimental Design and Diets. A 3 × 3 Latin square design was used (Supplementary Table 1). The study was divided into three experiments with experiment 1 investigating the effect of AF and MFX on both Borans (Experiment 1-Boran) and crosses (Experiment 1-crosses), experiment 2 (Experiment 2-Boran) studied the effect of FUM and FZYM on Borans and experiment 3 (Experiment 3-Boran) investigated the effects of combined AF and FUM on Borans. Each experiment comprised 6 animals divided into 3 groups of 2 animals each. At the start of the experiment, the animals were fed on a basal diet free from mycotoxins and detoxifiers for 5 weeks before feeding the experimental diets. The experiments were divided into 2 weeks of feeding experimental diets, followed by 4 weeks wash-out period where the animals were fed a basal diet free from mycotoxins and detoxifiers again. The groups within an experiment were then randomized for the order of the 3 treatments. The total duration of the animal trial was 23 weeks.

The basal diet of the animals was composed of good quality Rhodes grass hay and commercial dairy meal formulated according to nutrient requirements for dairy cows (Kenya Bureau of Standards, Supplementary Table 2). The hay and water were fed *ad libitum* while the dairy meal was divided into two and fed in the morning and evening. The amount of dairy meal fed was based on the average milk production, with Borans that are low producers receiving 2 kg of dairy meal per day, and crosses that produce higher levels of milk receiving 6 kg of dairy meal per day. This is in line with the practice done by small-scale dairy farmers in Kenya.[38] The daily doses of AFB1, FUM, and detoxifiers were formulated in the dairy meal.

The feed was formulated without the addition of any mycotoxin deactivation compound and used as the control feed. Both the dairy meal and hay had AF and FUM levels below the regulatory limit set at 10 μg/kg and 5000 μg/kg respectively[2] (i.e., 9 μg/kg and 104 μg/kg for dairy meal and < limit of detection (LOD) for hay, respectively). For the contaminated diets, AFB1 and FUM (FB1+FB2+FB3) culture material were prepared as a premix and mixed with the dairy meal to reach an average (mean ± SD) AFB1 level of 394 ± 18 μg/kg for experiment 1 on Borans, 385 ± 59 μg/kg for experiment 1 on crosses, and 476 ± 122 μg/kg for experiment 3. These levels were above the

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EU regulatory limit.[2] FUM level in the dairy meal was 6.2 ± 5.3 mg/kg for experiment 2 and 15.2 ± 4.4 mg/kg for experiment 3, which were below the EU guidance limit.[2] The levels of AFB1 and FUM after mixing were determined by LC−MS/MS using the method described by Sulyok et al.[39] The ratio of AFB1, AFB2, AFG1, and AFG2 was 92%, 5%, 2%, and <1%, respectively; hence, only AFB1 was considered. For groups requiring the addition of binder, the binder was mixed with the feed to have a level of 60 g/cow/day for MFX and 120 U/cow/day for FZYM.

The cows in experiment 1 received in random order AFB1 (788 μg/cow/day equivalent to 69.7 μg/kg DMI for Borans and 2,310 μg/cow/day equivalent to 154 μg/kg DMI for crossbreeds), bentonite (60 g/cow/day), or both AFB1 (788 μg/cow/day for Borans and 2,310 μg/cow/day for crossbreeds) and MFX (60 g/cow/day) for 2 weeks. This was followed by 4 weeks wash-out period where the animals were fed a basal diet before being assigned to the next treatment in the experiment until the animals had received all the 3 treatments.

In experiment 2, each cow received in random order FUM (12.4 mg/cow/day that is equivalent to 1.1 mg/kg DMI), FZYM (120 U/cow/day), or both FUM (12.4 mg/cow/day) + FZYM (120 U/cow/day) for 2 weeks. This was followed by 4 weeks wash-out period where the animals were fed a basal diet before being assigned to the next treatment in the experiment until the animals had received all the 3 treatments.

Each cow in experiment 3 received in random order AFB1 (952 μg/cow/day equivalent to 84.2 μg/kg DMI) + FUM (30.4 mg/cow/day equivalent to 2.7 mg/kg DMI), MFX (60 g/cow/day) + FZYM (120 U/cow/day) or both AFB1 (952 μg/cow/day) + FUM (30.4 mg/cow/day) and MFX (60 g/cow/day) + FZYM (120 U/cow/day) for 2 weeks. This was followed by a 4 weeks wash-out period where the animals were fed a basal diet before being assigned to the next treatment in the experiment until the animals had received all the 3 treatments.

2.4. Sample Collection and Analysis. *Milk.* Milk was collected before the start of feeding the contaminated feed and after the end of the feeding trial. The milk was collected by complete stripping of the udder and a 100 mL sample was collected and stored at −20 °C until it was analyzed for milk composition and transported for AF residues analysis. Milk composition analysis included protein, fat, solid nonfat (SNF), freezing point, and the total solids (TS) through infrared spectroscopy using Milkoscan FT1 (model MilkoScan FT1- FOSS, Hillerød, Denmark).

Blood. At the start and the end of each experimental period, 10 mL of blood samples were collected from the jugular vein into tubes with added anticoagulants and transported to the Regional Veterinary Investigation Laboratory, Kericho (Kenya) for analysis. The hematological parameters including white blood cell (WBC), red blood cell (RBC), hematocrit, hemoglobin levels, platelet count, and differential leukocyte counts were determined by the Mindray Auto hematology analyzer (Model BC-2800Vet, Shenzhen Mindray Bio-Medical Electronics Co. LTD, Shenzhen, China). Results were compared with bovine hematological reference ranges as described by Roland et al.[40]

For blood biochemistry and mycotoxin residue determination, blood from the jugular vein was collected into tubes without and with anticoagulants, respectively. The blood was then centrifuged at 3000*g* for 10 min, and serum was collected for blood biochemistry and plasma for AF residue and Sa/So ratio analysis. The plasma and serum samples were stored at −20 °C until analysis. Total bilirubin, ALP, AST, alanine aminotransferase, and GGT were determined by spectrophotometry (Biochrom WPA Lightwave II UV/visible spectrophotometer, UK) at the Regional Veterinary Investigation Laboratory, Kericho. Results were compared with bovine biochemical reference ranges as described by Lumsden et al.[41]

Rumen Fluid. At the start and end of each experimental period, rumen fluid was collected by oral stomach tube 2 h after morning feeding. The first 150 mL of the collected fluid was discarded to avoid saliva contamination. The rumen fluid was then stored at −20 °C until frozen transported for analysis for AF and FUM residues.

Fecal Samples. At the start and end of the experimental period, a fecal sample was collected using a gloved hand inserted in the rectum, and 200 g of feces were evacuated. The sample was then stored at −20 °C until frozen transported for analysis for FUM residues.

2.5. Determination of Aflatoxin Residues in Milk, Plasma, Rumen Fluid. Aflatoxin residues in milk, plasma, and rumen fluid were determined by UHPLC−MS/MS at the Department of Pathobiology, Pharmacology, and Zoological Medicine, Ghent University, Belgium, as described by De Baere et al. (submitted).[42] The LOD and limit of quantification (LOQ) in plasma were 0.002 and 0.025 ng/mL for AFB1 and AFM1, respectively, while the LODs for the different AF in milk and rumen fluid ranged from 0.0012−0.132 ng/mL and the LOQs from 0.1−0.5 ng/mL. The individual LODs and LOQs are shown in Supplementary Table 3.

2.6. Determination of Fumonisin Residues in Rumen Fluid. Fumonisin B1, B2, and B3 and their partially and completely hydrolyzed metabolites, HFB1, 2, 3, pHFB1a, 2a, 3a, and pHFB1b, 2b, and 3b were analyzed in rumen fluid based on the method as specified by Bartok et al.[43] Briefly, 400 μL of 1% formic acid in acetonitrile (ACN) was added to 200 μL of sample and vortexed for 5 s. The mixture was centrifuged at 19,000*g* for 10 min. The supernatant was then transferred to an HPLC vial and injected into the LC−MS/MS instrument. The LOQs were 2.10, 4.26, 1.17, 6.0, 12.12, 9.45, 1.29, 0.63, 1.47, 0.93, 0.75, and 0.63 ng/mL for FB1, FB2, FB3, HFB1, HFB2, HFB3, pHFB1a, pHFB2a, pHFB3a, pHFB1b, pHFB2b, and pHFB3b, respectively.

2.7. Determination of Sphingoid Bases in Plasma. Sphingolipids Sa and So were determined using LC−MS/MS using the method described by Schwartz-Zimmermann et al.[44] Briefly, 200 μL of plasma aliquots were shaken with 600 μL of methanol (MeOH)/ACN (50/50, v/v) for 30 min, then centrifuged at 14,000*g*. This was followed by pellet extraction done with 300 μL of MeOH/water (80/20, v/v), and followed by centrifugation. The supernatant was then dried, and the resulting residue was reconstituted in 300 μL of ACN/water (30/70, v/v) and centrifuged before LC−MS/MS analysis. The LOQ of Sa and So was 1.5 ng/mL in plasma.

2.8. Determination of Fumonisin Residues in Feces. The concentration of FB1, 2, and 3, their partially and completely hydrolyzed metabolites, was determined by LC−MS/MS using the method previously described by Masching et al.[45] Briefly, 1 g of lyophilized feces was weighed into falcon tubes and ACN/water/formic acid (74/25/1, v/v/v) was added and then vortexed. This was followed by centrifugation for 5 min at 1880*g*. The same procedure was repeated two more times but with centrifugation for 10 min at 3200*g*. The aliquots of the combined supernatants were then diluted 1:1 with extraction solvent and then injected into the LC−MS/MS instrument. The LOQs were 0.70, 2.00, 1.42, 0.39, 4.04, 3.15, 0.31, 0.43, 0.25, 0.21, 0.21, and 0.49 μg/g for FB1, FB2, FB3, HFB1, HFB2, HFB3, pHFB1a, pHFB2a, pHFB3a, pHFB1b, pHFB2b, and pHFB3b, respectively.

2.9. Calculations. Milk AFM1 transfer variables were calculated as follows:

$$\text{Excretion } (\mu g/d) = \text{AFM1 concentration in milk } (\mu g/L) \times \text{milk yield on sampling day } (L/d)$$

$$\text{Carry-over } (\%) = \frac{\text{excretion of AFM 1 } (\mu g/d)}{\text{AFB1 consumption } (\mu g/d)} \times 100$$

Individual animal carry-over was calculated based on the AFM1 concentration and the cow's milk production which was then used to calculate the group means. Milk production was measured by complete stripping of the milk during the sampling days, and not allowing the calves to suckle.

2.10. Data Analysis. Each animal acted as its control and measurements were taken before and after the treatment. The change (measurement after the treatment subtracted from measurement before treatment) was used for comparison. For data analysis purposes, half the LOD was used for samples that had levels below the LOD, and hence nothing was measured, while for samples with

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detectable levels but below LOQ half the LOQ was used. We fitted a linear model (estimated using OLS) to predict the response variable with Sequence, ID, Treatment, Period, and Lactationstage (formula: Response variable ~ Sequence + Sequence:ID + Treatment + Period + Lactationstage). The sequence was included in this model to check for evidence of carry-over effect from the previous treatment through the sequence as described by Lim et al.[46] The lactation stage was divided into three periods; early (1−100 days), mid (101−200 days), and late lactation (>200 days),[47] while period was divided into the three treatment periods 1, 2, and 3. No significant carry-over was detected which is an indication that the wash-out period between treatments was adequate. The results were reported as least-squares means (n = 6 for each experiment) to adjust for the means of other factors in the model.

3. RESULTS

3.1. Feed Intake, Milk Yield, and Milk Composition. Dry matter intake (DMI) and milk yield were not affected by the treatments with the DMI (mean ± SD) averaging 11.3 ± 1.4 and 15.0 ± 1.3 kg for Borans and crosses, respectively, and milk yield is shown in Supplementary Table 4. Feeding the mycotoxins with or without the detoxifiers also did not affect the milk composition as shown in Supplementary Table 4.

3.2. Blood Hematology and Biochemistry. *Hematological Parameters.* All the hematological parameters tested were within the clinical range in cattle for all the experiments before and after the treatments. No significant change in the hematological parameters was observed among the groups in experiment 1 and experiment 3. In experiment 2, the addition of FZYM alone (-1.65×10^{-9}/L) led to a significant drop in granulocytes number (p = 0.04) as compared to FUM alone (-0.18×10^{-9}/L) but not for the combination of FUM and FZYM (-0.65×10^{-9}/L) (Supplementary Table 5).

Blood Biochemistry Parameters. The biochemical parameters before and after treatment were within the normal clinical range for lactating cattle.

Treatment with AF, FUM, MFX, or FZYM either singly or combined had no clinically significant effect on ALT, AST, ALP, bilirubin, and GGT with the parameters within the normal ranges. However, in the FUM treatment experiment, there was a significant decrease in ALT in the FUM treatment group (−9.8 u/L) as compared to the FZYM group (7.8 u/L) which had an increase (p = 0.03), but no significant difference in the combined FUM+FZYM group (1.0 u/L) (Supplementary Table 6).

3.3. Aflatoxins in Milk, Plasma, and Rumen Fluid. *Aflatoxins in Milk.* Table 1 and Supplementary Table 7 show the different classes of AF in milk for the various treatment groups. Table 2 further shows the excretion and carry-over rate. Milk collected before the start of the treatment had no detectable levels of AFB1, AFB2, AFG1, and AFG2, while the mean levels of AFM1 and AFM2 for Borans (0.04 μg/L and 0.05 μg/L respectively) and crosses (0.13 μg/L and 0.05 μg/L, respectively) were low. Following treatment, there was a significant difference in AFB1 and AFM1 levels in AF-treated groups as compared to groups with only the detoxifiers given. Crosses also had a higher carry-over rate (0.35%) as compared to Borans (0.08%). The addition of MFX in feed with AFB1 resulted in a significant drop in mean AFM1 concentration for both Borans (0.689 vs 0.345 μg/L; p = <0.001) and crosses (2.74 vs 1.49 μg/L; p = 0.001) which is equivalent to a milk AFM1 concentration decrease of 30% and 43% in Borans and crosses, respectively. The carry-over also decreased to 0.20%

Table 1. Least Mean Squares of Changes in Aflatoxin B1 and M1 Concentrations (μg/L) in Milk, Plasma, and Rumen Fluid Following Treatments with Aflatoxins with or without Fumonisins, Bentonite, and Fumonisin Esterase[a]

	expt 1 Boran (n = 6)					expt 1 crosses (n = 6)					expt 3 Boran (n = 6)				
	AF	MFX	AF+MFX	SEM	P	AF	MFX	AF+MFX	SEM	P	AF+FUM	MF+FZYM	AF+FUM+ MFX+FZYM	SEM	P
Milk															
AFB1	0.078a	0.008b	0.051ab	0.018	0.02	0.116a	0.004b	0.061c	0.017	0.003	0.094a	0.010b	0.110a	0.381	0.04
AFM1	0.689a	−0.065b	0.345c	0.123	<0.001	2.737a	−0.010b	1.493c	0.323	0.001	1.126a	−0.068b	0.956a	0.454	0.04
Plasma															
AFB1	0.028a	<0.001b	0.022a	0.007	0.02	0.046a	0.004b	0.024c	0.004	<0.001	0.043a	0.008b	0.057a	0.01	0.003
AFM1	0.025a	0.007b	0.011a	0.01	0.03	0.096a	0b	0.056c	0.007	<0.001	0.046a	0.001b	0.045a	0.012	0.01
Rumen fluid															
AFB1	0.304a	0.004b	0.318a	0.115	0.03	0.628a	0.02b	1.24a	0.284	0.04	0.566	0.144	0.9	0.327	0.12
AFM1	ND	ND	ND	ND		0.022	0	0.020	0.013	0.20	0.018	0.01	0.025	0.007	0.20

[a]AF − aflatoxins treatment group; AF+MFX − aflatoxins plus bentonite treatment; AF+FUM − aflatoxins and fumonisins treatment; AF+FUM+MFX+FZYM − aflatoxins, fumonisins, bentonite, and fumonisin esterase treatment; MFX − bentonite treatment group; ND − below the limit of detection both before and after treatment; P − P value due to treatment; a,b,c − groups with different letters denote a significant difference between the groups; SEM − standard error of means. Note: Negative value indicates a decrease after treatment compared to before treatment.

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Table 2. Aflatoxin M1 Concentration, Excretion, and Carry-over in Milk for Treatments with Aflatoxins with or without Fumonisins, Bentonite, and Fumonisin Esterase[a]

	expt 1 Boran (n = 6)				expt 1 crosses (n = 6)				expt 3 Boran (n = 6)			
	AF	MFX	AF +MFX	P	AF	MFX	AF +MFX	P	AF +FUM	MFX +FZYM	AF+FUM+ MFX +FZYM	P
AFB1 intake (μg/cow/day)	788	0	788	NA	2,310	0	2,310	NA	952	0	952	NA
AFM1 concentration (μg/L)	0.86b	0.02a	0.60b	<0.001	2.87b	0.12a	1.65c	<0.001	1.22b	0.03a	0.693c	<0.001
AFM1 excretion (μg/day)	0.61b	0.01a	0.50b	<0.001	8.19b	0.46a	4.63c	<0.001	0.52b	0.01a	0.23c	<0.001
Carry-over %	0.08	NA	0.06	0.56	0.35	NA	0.20	0.13	0.05	NA	0.02	0.07

[a]AF − aflatoxins treatment group; AF+MFX − aflatoxins plus bentonite treatment; AF+FUM − aflatoxins and fumonisins treatment; AF+FUM +MFX+FZYM − aflatoxins, fumonisins, bentonite, and fumonisin esterase treatment; MFX − bentonite treatment group; NA − Not applicable; P − treatment effect p-value; a,b,c − groups with different letters denote significant difference exists among the groups.

Table 3. Least Square Means of Change in Sphinganine, Sphingosine Concentration, and Their Ratio in Plasma Following Fumonisins Treatment[a]

	expt 2 Boran (n = 6)					expt 3 Boran (n = 6)				
	FUM	FZYM	FUM+ FZYM	SEM	P	AF+ FUM	FZYM+ FUM	AF+FUM+ MFX+FZYM	SEM	P
Sa (μg/L)	0.32	−0.94	−1.13	1.34	0.68	−0.22	−0.70	−1.93	1.58	0.56
So (μg/L)	1.53	−2.74	−3.07	−4.83	0.72	−0.02	−1.91	−6.22	5.23	0.51
Sa/So	0.04	−0.08	−0.10	0.09	0.48	−0.07	−0.18	−0.07	0.05	0.12

[a]AF+FUM − aflatoxins and fumonisins treatment; AF+FUM+MFX+FZYM − aflatoxins, fumonisins, bentonite, and fumonisin esterase treatment; FUM − fumonisins treatment group; FZYM − fumonisin esterase treatment group; FUM+FZYM − fumonisins and fumonisin esterase group; Sa − Sphinganine; So − Sphingosine, Sa/So − Sphinganine/Sphingosine; P value − P value due to treatment at a significant level of 0.05. Note: Negative value indicate a decrease after treatment compared to before treatment.

Table 4. Least Square Mean of the Change in Concentration of Fumonisins and Fumonisin Metabolites (HFBs, pHFBa, and pHFBb) in Rumen Fluid (μg/L) and Feces (μg/g) Following Treatment with Fumonisins and/or Fumonisin Esterase[a]

	expt 2 Boran (n = 6)					expt 3 Boran (n = 6)				
	FUM	FZYM	FUM+ FZYM	SEM	P value	AF+FUM	BEN+ FZYM	AF+FUM+ MFX+FZYM	SEM	P value
Rumen fluid										
FBs	154.6a	−45.7b	105.6a	51.4	0.04	330.9a	−37.1b	130.1c	61.9	0.001
HFBs	−1.11a	−4.91a	10.78b	4.47	0.04	−3.35	−2.0	10.43	7.01	0.12
pHFBa	0.83ab	−1.31a	3.31ab	1.23	0.04	3.31a	1.14b	3.74a	1.55	0.04
pHFBb	2.23a	−2.06a	16.74b	3.05	0.003	2.84a	−1.27a	16.20b	5.18	0.01
Feces										
FBs	2.70	1.21	2.41	0.42	0.18	5.08 a	ND	2.84b	0.27	<0.001
HFBs	ND	ND	1.02	0.01	0.001	ND	ND	1.23	0.14	0.08
pHFBa	0.04a	0.03a	0.13b	0.02	0.02	0.12	0.03	0.11	0.03	0.06
pHFBb	NDa	NDa	0.32b	0.06	0.004	0.16 ab	0.03a	0.29b	0.06	0.01

[a]a,b,c − group with different letters denotes difference among groups; AF+FUM − aflatoxins and fumonisins treatment; AF+FUM+MFX +FUMZYM − aflatoxins, fumonisins, bentonite, and fumonisin esterase treatment; FUM − fumonisins treatment group; FZYM − fumonisin esterase treatment group; FUM+FZYM − fumonisins and fumonisin esterase group; HFBs − hydrolyzed fumonisins; pHFB − partially hydrolyzed fumonisins; P value − p-value due to treatment. Note: Negative value indicates a decrease after treatment compared to before treatment.

and 0.06% for crosses and Borans, respectively, though this was not statistically significant.

A similar pattern was observed for the treatment with both AFB1 and FUM (experiment 3), with a 43% decrease in milk AFM1 concentration (1.13 vs 0.96 μg/L) following treatment with both MFX and FZYM and a numerical decrease in carry-over from 0.05% to 0.02%.

Aflatoxins in Plasma. The levels of the different AF in plasma are shown in Table 1 and Supplementary Table 7. A significantly higher level of AFB1 and AFM1 was detected in groups fed AF-contaminated feed as compared to groups with only detoxifiers (MFX and FZYM). The addition of MFX in the AFB1-contaminated diet led to a decrease in AFM1 and AFB1 in all groups as compared to the AFB1-only treatment, but a significant difference was only seen for AFB1 (0.046 vs 0.024 μg/L; $p < 0.001$) and AFM1 (0.096 vs 0.056 μg/L; $p < 0.001$) in experiment 1 with the crosses.

Aflatoxins in Rumen Fluid. The concentration of different classes of AF in rumen fluid is shown in Table 1 and Supplementary Table 7. AFB1 was the main AF found in rumen fluid following feeding with AF-contaminated feed with AFB2 and AFM1 being below the LOD or detected in low levels. Neither AFG1 nor AFG2 was detected. The groups fed with AF-contaminated feed had a significantly higher level of AFB1 as compared to groups with only detoxifiers. Higher levels of AFB1 were detected following the addition of MFX in the AFB1-contaminated diet, but this was not statistically

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different. AFM1 and AFM2 were only detected at lower levels in groups given AF.

3.4. Fumonisins and Biomarkers in Plasma, Rumen Fluid, and Feces. *Biomarkers in Plasma.* Table 3 shows the mean change in Sa and So and their ratio in plasma. Following the feeding of FUM either alone or in combination with AF or FZYM, there was no significant change in the Sa/So ratio between the groups.

Biomarkers in Rumen Fluid. An overview of the mean of FUM and its metabolites is presented in Table 4. Groups that received FUM in feed alone or with AF, had a significantly higher increase in FUM level (154.6 and 330.9 μg/L respectively) in the rumen fluid as compared to groups with only detoxifiers. On the other hand, fully hydrolyzed FUM (HFBs) and partially hydrolyzed FUM (pHFBs) remained low in groups with no FZYM added. Following the addition of FZYM in feed contaminated with FUM, there was a decrease in FUM level and an increase in the level of HFBs and pHFBs. For pHFBs, pHFBb was more abundant as compared to pHFBa. A similar result was obtained in the fecal samples with HFBs only present in groups receiving combined FUM and FZYM and an increase in pHFBs in the same groups.

4. DISCUSSION

The present study investigated the effects of two mycotoxins on animal health and productivity as well as on AFM1 carry-over to milk. Further, the efficacy of the addition of an adsorbent (Mycofix Secure) at the manufacturer's recommended rate of 60 g/cow/day and an enzyme (FUM*zyme*) at a rate of 120 U/cow/day was assessed. No cow displayed any clinical signs of mycotoxicosis during the period of the study, which is in line with previous studies using almost similar or lower mycotoxin levels.[25,31]

Dry Matter Intake and Milk Composition. The inclusion of the adsorbent and/or the enzyme in the feed contaminated with AF and/or FUM does not affect DMI or milk composition, as reported by several studies using AFB1 at levels between 20−112 μg/kg DMI and up to 2% clay-based adsorbents.[19,27,31] This is in line with the findings of this study, however, Sulzberger et al.[22] reported a negative linear treatment effect for milk yield and a quadratic treatment effect for DMI in cows supplemented with clay as compared to cows with no clay and with 100 μg/kg DMI AFB1. For FUM, Gallo et al.[11] reported no change in the DMI on cows fed 1,247 μg of FUM/kg of DMI with the addition of 35 g/cow/day Mycofix, which is in line with our findings. The results in the current study, therefore, demonstrate that the addition of a detoxifier in feed with AF and/or FUM did not influence the lactation performance of dairy cows.

Hematological and Blood Biochemical Parameters. Hematological and biochemical parameters can be used for the early detection of aflatoxicosis before the clinical symptoms occur.[18,20] A decrease in erythrocytes, leucocytes, hemoglobin, and hematocrit has been reported due to feeding between 75−250 μg/kg AFB1 for 2 weeks, but these levels were still within the normal ranges.[20,48] However, in the present study, no effect on the hematological parameters was observed. Additionally, plasma GOT, GPT, GGT, and ALP have been suggested as indicators of depressed liver function such as due to chronic AF and FUM exposure.[19,23] No effect on blood biochemical parameters was reported in the present study following AF exposure, which is consistent with findings by Xiong et al.[19] A significant decrease in ALT and granulocyte numbers following FUM treatment as compared to giving only the enzyme or FUM in combination with the enzyme was observed, but the levels were within the normal clinical range in cattle. The lack of effect in our study may be attributed to the low dose of mycotoxins used and the relatively short duration of exposure (14 days), and the ability of the rumen to break down AF and FUM may have rendered the mycotoxins relatively harmless, as suggested by Wang et al.[18]

Carry-over of AFB1 from Feed to AFM1 in Milk. As expected, feeding AFB1 showed a significant food safety concern through AFM1 carry-over to milk. Aflatoxin M1 secretion and carry-over to milk are influenced by several factors with the level of milk production and the presence of other mycotoxins being among them.[25,26,49] An exponential increase in carry-over with milk yield has been described previously.[25,27,50] All the cows used in this study had a very low milk yield compared to studies done in high-income countries. Friesian-Boran crossbreeds, which had a higher milk production (average 3.7 L/day), had a modestly higher level of AFM1 carry-over (0.35%) as compared to Boran (0.08%) that had a lower milk production (average 0.77 L/day). Our finding in Borans is in agreement with the formula by Britzi et al.[25] for carry-over, that is, % = 0.5154 ^{e}(0.0521) × milk yield. However, using this formula would give a somewhat lower level (0.27% vs the measured 0.35%) for the Friesian-Boran crossbreeds. Synergistic and additive health effects of AF and FUM in pigs, chickens, and humans have previously been reported,[13,28,51] but despite no significant difference being observed in the present study, more research needs to be done on the impact of FUM on the carry-over of AFM1 to further explore if there is any impact, and if so, what are the mechanisms. The reduction in carry-over of AFM1 in milk following the addition of adsorbent can most likely be attributed to the binding of AFB1 and the adsorbent making AFB1 unavailable for absorption. Bentonite (dioctahedral montmorillonite) is the main ingredient in the Mycofix Secure, a silicate material. The polarity of AFB1 enhances its binding or adsorption by these silicate materials thus making them unable to be absorbed into circulation from the gastrointestinal tract.[37,52] Previous studies have reported a similar reduction in milk AFM1 concentration and carry-over following the addition of an adsorbent in contaminated feed. Kutz et al.[31] reported a 48% decrease in cows fed 112 μg/DMI AFB1 with 125 g/cow/day of an adsorbent (NovasilPlus) that contained hydrated sodium calcium aluminosilicates as the main ingredient. Pietri et al.[30] used 50 g/cow/day and 20 g/cow/day of a bentonite-based commercial detoxifier that also had Eubacterium and yeast. Kissell et al.[32] used bentonite at a level of 227 g/cow/day, and Sulzberger et al.[22] using 0.5%, 1%, and 2% of clay containing vermiculite, nontronite, and montmorillonite showed a reduction of between 25% and 60% of AFM1 carry-over to milk from cows fed on feed contaminated with between 97.3 and 112 μg of AFB1/kg DMI. On the other hand, Sumantri et al.[27] used bentonite at an inclusion rate of 0.25% and 2% in the diet of cows containing 350 μg AFB1/cow/day for 5 days, and Ogunade et al.[48] used 20 g/cow/day of an adsorbent containing sodium bentonite and *S. cerevisiae* fermentation product in feed with 75 μg of AFB1/kg DMI, and reported no reduction in AFM1 carry-over. However, the latter authors reported shortening of the time required to achieve safe levels of AFM1 in milk following withdrawal of AFB1 contaminated feed. In goats, Mugerwa et al.[53] reported a reduction of AFM1 carry-over in milk following the addition of

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1% DMI activated charcoal and calcium bentonite in feed with 100 μg of AFB1/kg. From the findings, the ability of bentonite in reducing the carry-over of AFM1 in milk was evident. Despite the levels not coming down to the East Africa Community (EAC) and EU AFM1 regulatory limit in milk of 0.5 and 0.05 μg/kg, respectively, it should be noted that the level of AFB1 used for the study (385−476 μg/kg) was almost 80-fold the EU and the EAC regulatory limit and much higher than what most studies have established. However, such levels have been reported to occur in dairy feeds in SSA.[2] This, therefore, means at lower AFB1 levels bentonite will be useful in reducing AFM1 levels in milk to below the limits and hence can be used by dairy farmers in their feed.

Aflatoxins and Fumonisins Metabolites in the Rumen Fluid. The rumen plays a vital role in AFB1 detoxification explaining partly the low levels in milk as compared to the consumed AFB1 level. Following ingestion by ruminants, part of the AFB1 undergoes degradation in the rumen. *Lactobacillus spp* and *Streptococcus spp* are facultative anaerobes in the rumen and have been shown to degrade AFB1 to metabolites such as aflatoxicol (AFL), the less toxic metabolite aflatoxin B2a (AFB2a), and the nontoxic metabolite aflatoxin D1 (AFD1).[54] This, therefore, leads to a reduction in the level of AFB1 available for absorption. Further, the addition of the adsorbent in the contaminated diet leads to the binding of AFB1 with the adsorbent reducing its bioavailability. On the other hand, in the presence of FUM, the addition of both the adsorbent and enzyme led to a higher reduction of 43% in AFM1 concentration in milk. Previous studies have shown the efficacy of FZYM in cattle.[11,33] This was confirmed in our study where the addition of the enzyme in feed contaminated with FUM led to a statistically significant increase in the level of HFBs and pHFBs both in rumen fluid and feces. It can also be noted that no or low level of HFBs and pHFBs was present in the group with FUM alone or in combination with AFB1 without the addition of FZYM, indicating the ability of the rumen to degrade small quantities of FUM, as previously reported by Gurung et al.[55]

Aflatoxins and Fumonisins Biomarkers in Plasma. Following absorption, AFB1 is converted into AFB1-lysine adduct, AFM1, aflatoxin Q1 (AFQ1), and AFL. AFL acts as a reservoir of AFB1 and can be reconverted back to AFB1 which can be detected in plasma, while AFM1 and AFQ1 are not metabolized further and are excreted in urine and milk.[6,7,56] This adduct and metabolites can therefore be used as biomarkers of AFB1 exposure. The addition of adsorbent in the AFB1 contaminated feed led to a lower level of AFB1, AFG1, and AFM1 indicating the adsorbent's ability to reduce the bioavailability of AF and hence prevent adverse effects of AF. For FUM, the Sa/So ratio is the most relevant biomarker for high acute FB1 exposure in plasma. In the present study, no significant change was observed in the Sa/So ratio following feeding with FUM alone or in combination with the enzyme. On the contrary, Baker and Rottinghaus[23] reported elevated Sa/So ratios with mild changes in the hepatocellular and bile duct epithelial morphology in calves. The calves were fed a diet contaminated with 2.36 mg/kg/day FB1 increased to 3.54 mg/kg/day for 239 to 253 days. This, therefore, indicates that the Sa/So ratio may not be a good biomarker for a low-non toxic level of FUM exposure below the EU guidance limit in dairy cattle, and a longer period may be required for observable changes in ruminants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c08217.

Supplementary Tables 1−7 show the study design, chemical composition of basal diet, LC−MS/MS limit of detection and quantification of different aflatoxin (AF) metabolites, results of hematological and blood biochemical parameters and results of the different AF metabolites in milk, plasma and ruminal fluid. (PDF)

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Notes

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