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Effects of supplemented multicomponent mycotoxin detoxifying agent in laying hens fed aflatoxin B1 and T2-toxin contaminated feeds

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ABSTRACT The present study was conducted to determine the ability of multicomponent mycotoxin detoxifying agent (**MMDA**) in feed to prevent the gastrointestinal absorption of aflatoxin B1 (AFB1) and T2-toxin supplemented via spiked maize. For comparisons, hens were fed with uncontaminated basal diet without or with addition of MMDA at 2 g/kg feed. The trial consisted of 105 laying hens (Lohmann Brown) without obvious signs of disease allocated to 7 treatment groups in 35 pens. Responses were demonstrated on laying performance and health status throughout the 42 d experimental period. The results of laying performance indicated significantly decreased egg mass with increasing mycotoxin (AFB1 and T2-toxin) levels up to the maximum tolerated dosage, however simultaneous presence of MMDA laying performance was slightly modified linearly to increasing application. Dose-dependent pathological changes in liver and kidneys and their relative weights, changes in blood parameters and reduced eggshell weights were observed in the hens fed AFB1 and T2-toxin. The pathological changes in the hens fed with diets containing AFB1 and T2-toxin without MMDA were significantly higher as compared with the control group, but eggshell stability was not affected. The contents of AFB1, T2-toxin and their metabolites in liver and kidney tissues were significantly decreased in the hens supplemented with MMDA at 2 and 3 g/kg in feed. MMDA supplementation significantly reduced the deposition of AFB1, T2-toxin and their metabolites in liver and kidneys at the maximum tolerated dosage (2 and 3 g/kg) indicating specific binding to AFB1 and T2-toxin in the digestive tract as compared to the corresponding diets without MMDA. Exposure of AFB1 and T2-toxin indicated significantly decreased egg mass with increasing mycotoxin levels up to the maximum tolerated dosage because of the significantly reduced egg production. Therefore, in this study, MMDA could reduce negative effects of feeding AFB1 and T-2 to laying hens.

Key words: mycotoxin, laying hen, aflatoxin B1, MMDA, T2-toxin

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INTRODUCTION

Mycotoxins are harmful secondary metabolites originated from filamentous fungi and poses several detrimental effects to human and animal health (Warth et al., 2016; Alshannaq and Yu, 2017; Eskola et al., 2020). The cereal crops in agricultural practices may be contaminated with fungal toxins during harvesting, transport, processing and storage and their production is influenced by several climatic factors (Coffey et al., 2009; Warth et al., 2016). Apart from the alarming deteriorative health effects produced by mycotoxins, animal derived products such as meat, milk, and eggs carry over the mycotoxins and does produce an impact on human health as well (Alshannaq and Yu, 2017). An around more than 200 mycotoxins producing species of molds are already known and the most common harmful mycotoxins contaminating the feed stuff and feed material include AFB1 (**AFB1**), HT-2, T-2, ochratoxin A, fumonisin B1, zearalenone, citrinin, etc. (Binder, 2007).

Aflatoxins in feed are reported to produce severe losses in terms of mortality and production losses. The metabolites of aflatoxins are very hardy and are not even destroyed after processing. There are several forms of

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aflatoxins produced in poultry namely AFB1, B2, G1 and G2 produced by fungi *Aspergillus favus* and *Aspergillus parasiticus* (Arafa et al., 1981). AFB1 is most potent known among these and is reported to be transferred from feedstuff given to the poultry to eggs, meat and other edible parts of the poultry carcass (Pitt and Miller, 2017; Rajput et al., 2017; Liu et al., 2018; Saminathan et al., 2018). Aflatoxicosis in layer birds is concerned with reduced egg weight and production, decline in body weight, poor feed conversion ratio (**FCR**), fatty liver, immunosuppression, and reduction in the levels of enzymes related with the digestion of lipids, proteins and starch (Edds and Bortell, 1983; Leeson et al., 1995; Devegowda and Murthy, 2005). T-2 is another important mycotoxin linked to declined growth, decreased egg production and quality, leucopenia, regression in bursa of Fabricius, defective blood coagulation, and immunosuppression in poultry birds (Leeson et al., 1995; Dänicke et al., 2001). T2 and ochratoxin are the commonly known mycotoxins present in poultry feed and work synergistically with aflatoxins (Huff and Doerr, 1981; Huff et al., 1988a).

To limit the effect of mycotoxins several strategies are tried to counteract the detrimental impacts of mycotoxins. The strategies to prevent the absorption of mycotoxins must be economical and should not produce any harmful effects through the deposition of their residues in the feed and tissues and simultaneously should not create any deteriorating effect on the nutritional quality of feed products (Parlat et al., 1999). Addition of clinoptilolite at the rate of 15 g/kg has shown protective effect against 2,500 μg/kg aflatoxin in feed (Oguz and Kurtoglu, 2000). According to 1 study's findings, chicks given AFB1-contaminated diets at 2,500 μg/kg can effectively boost bodyweight gain and partially recover from aflatoxicosis using hydrated sodium calcium aluminosilicate at dose of 5 g/kg (Chen et al., 2014). Absorbent bentonite (7.5 g/kg at 2,000 μg/kg aflatoxin dose) into the AFB1 diet diminished the effects of AFB1 and a substantial drop in the amount of AFB1 residues found in avian liver (Dos Anjos et al., 2015; Bhatti et al., 2018). Nano-composite magnetic grapheme oxide with chitosan (5 g/kg at 22 μg/kg aflatoxin dose) produced pronounced reduction in the aflatoxins in gastrointestinal tract of birds and effectively increased overall performance (Saminathan et al., 2018). Yeast cell wall (1.5 g/kg at 350 μg/kg aflatoxin dose) improved weight gain and feed conversion rate (Mendieta et al., 2018). Probiotic (1 g/kg at 250 μg/kg aflatoxin dose) substantially lowered liver aflatoxin concentrations and neutralized the harmful effects of AFB1 (Salem et al., 2018). Alpha-lipoic acid (300 g/kg at 300 μg/kg aflatoxin dose) reduce tissue damage brought about by AF in the chicks' kidney and liver (Karaman et al., 2010). *Urtica diocia* seed extract (300 g/kg at 1,000 μg/kg aflatoxin dose) exhibited a hepatorenal protective effect in birds, possibly acting via enhancing the cellular antioxidant mechanisms (Uyar et al., 2016). Grape seed proanthrocyanidin extract (250 g/kg at 1,000 μg/kg aflatoxin dose) significantly reduced AFB1 residues in liver and proacted against AF induced damage (Rajput et al., 2017). Curcuminoids (74 mg/kg at 1000 μg/kg aflatoxin dose) produced increase in feed intake and weight gain, and abated relative liver weight (Gowda et al., 2008).

Researchers have studied several mycotoxin binding materials to hamper the absorption of aflatoxins into blood circulation (Abo-Norag et al., 1995; Rosa et al., 2001). One in vivo experimental study in broiler birds has reflected the absorbent nature of smectite clay and the binding ability is attributed to the ion exchange capability, high surface area and swelling behavior in the vicinity of water (Manafi, 2012). The use of smectite-based mycotoxin binder is proven to improve the humoral immune response, growth performance and reduction in harmful toxicological effects on liver in broiler birds administered with AFB1 (Zabiulla et al., 2021). In one of the studies conducted has proven the ameliorative effect of multicomponent mycotoxin detoxifying agent (**MMDA**) against AFB1 and ochratoxin A in broiler birds (Tsiouris et al. 2021).

Multicomponent mycotoxin detoxifying agent a product from PATENT CO DOO. (Misicevo, Serbia), contains modified zeolite (Clinoptilolite), *Bacillus subtilis, Bacillus licheniformis, Saccharomyces cerevisiae* cell wall and silymarin in-feed, to reduce gastrointestinal absorption of AFB1 and T-2 toxin in broilers. The present study was performed to evaluate the effects of MMDA, on various performance parameters, egg characteristics, blood profile and gross examination of various organs in layer birds exposed to AFB1 and T-2 toxins.

RESULTS AND DISCUSSION

Clinical Symptoms and Effect on Overall Laying Performance of Hens

Laying hens were healthy during the study and conditioning scores reached a scale of 1 indicating normal activity and alertness, normal coat, and eyes. Consistency of excreta was within the physiological range; dry matter content of excreta reached approximately 30% and did not differ across treatment groups. Therefore, neither MMDA nor AFB1 and T2-toxin at 2 dose levels each did show any treatment related clinical sign of hens during the 42-d experimental feeding period.

The analyzed nutrients and mycotoxin concentrations in the experimental diets were in line with the calculated contents within the accepted tolerances. Furthermore, the reported ash contents indirectly confirm the expected dose levels of MMDA. Feeding AFB1 and T2-Toxin via spiked maize at the guidance dose level without MMDA (T3) showed significantly (overall egg production: -7.7%; overall egg mass: -6.8%) or slightly negative impacts on laying performance (overall feed intake: -2.9%; overall FCR: +4.3%) in comparison with the control group. Application of MMDA at 1 g/kg feed to diets containing AFB1 at 0.05 mg/kg feed in combination with T2-toxin at 1.5 mg/kg feed (T4) resulted in slightly positive modifications as compared with hens

Table 1. Effects of MMDA on laying performance of hens from d 15 to d 28 on trial (d 246 to d 259 of age).

Treatment groups		T1	T2	T3	T4	T4	T6	T7	Oneway ANOVA
Hens	n°	15	15	15	15	15	15	15	*P*
Replicates	n°	5	5	5	5	5	5	5	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
d 15 to d 21 on trial (d 246 to d 252 of age)									
Body weight - end	g	1992.4 ± 53.5	1967.8 ± 102.8	2011.8 ± 58.0	1963.0 ± 35.3	1945.8 ± 101.7	1968.0 ± 71.2	1957.2 ± 38.2	0.799
Body weight change	g	1.6 ± 18.2	4.8 ± 33.4	22.6 ± 13.0	25.6 ± 8.8	9.6 ± 30.7	-2.2 ± 27.5	7.0 ± 28.1	0.497
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Egg production	n°	6.9 ± 0.2^{c}	6.8 ± 0.4^{bc}	5.9 ± 0.4^{ab}	6.3 ± 0.4^{abc}	5.9 ± 0.7^{a}	6.1 ± 0.5^{abc}	6.1 ± 0.5^{abc}	**0.008**
Egg weight	g	62.9 ± 1.7^{a}	64.5 ± 0.9^{ab}	65.4 ± 1.0^{b}	64.5 ± 0.7^{ab}	64.4 ± 0.6^{ab}	63.9 ± 0.6^{ab}	64.6 ± 0.9^{ab}	**0.028**
Egg mass	g	432.0 ± 10.2^{ab}	438.7 ± 27.3^{a}	388.0 ± 29.8^{ab}	404.3 ± 22.3^{ab}	377.7 ± 43.8^{b}	392.1 ± 31.3^{ab}	395.8 ± 29.8^{ab}	**0.020**
Feed conversion ratio[1]		2.007 ± 0.038	1.942 ± 0.226	2.175 ± 0.207	2.075 ± 0.164	2.214 ± 0.235	2.136 ± 0.171	2.122 ± 0.246	0.340
Broken eggs	n°	0.4 ± 0.5	0 ± 0	0 ± 0	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0 ± 0	0.516
Dirty eggs	n°	0.2 ± 0.4	0.6 ± 0.9	0.4 ± 0.5	0.2 ± 0.4	0 ± 0	0.2 ± 0.4	0 ± 0	0.482
d 22 to d 28 on trial (d 253 to d 259 of age)									
Body weight end	g	1996.2 ± 61.2	1963.6 ± 94.7	1992.6 ± 58.7	1978.8 ± 43.6	1945.0 ± 73.1	1967.4 ± 67.8	1965.8 ± 57.6	0.903
Body weight change	g	3.8 ± 39.4	-4.2 ± 19.0	-19.2 ± 14.8	15.8 ± 25.4	-0.8 ± 33.7	-0.6 ± 13.1	8.6 ± 21.6	0.491
Feed intake	g	868.4 ± 11.7	858.2 ± 16.9	862.4 ± 18.1	869.8 ± 15.9	861.0 ± 45.1	875.6 ± 24.6	867.1 ± 19.3	0.927
Egg production	n°	7.0 ± 0.2	6.9 ± 0.4	6.6 ± 0.4	6.7 ± 0.4	6.3 ± 0.6	6.5 ± 0.5	6.5 ± 0.4	0.143
Egg weight	g	65.1 ± 1.8	65.0 ± 1.0	65.4 ± 1.3	64.9 ± 1.8	65.4 ± 1.6	65.2 ± 0.7	65.3 ± 1.2	0.996
Egg mass	g	456.0 ± 19.6	450.7 ± 23.4	431.9 ± 29.2	437.3 ± 32.2	414.2 ± 36.3	422.1 ± 36.3	426.4 ± 22.8	0.275
Feed conversion ratio[1]		1.907 ± 0.094	1.910 ± 0.130	2.006 ± 0.174	1.999 ± 0.173	2.093 ± 0.231	2.090 ± 0.232	2.038 ± 0.118	0.460
Broken eggs	n°	0.2 ± 0.4	0 ± 0	0.2 ± 0.4	0.4 ± 0.5	0.2 ± 0.4	0.2 ± 0.4	0.4 ± 0.5	0.820
Dirty eggs	n°	0.4 ± 0.5	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.991

Abbreviations: AFB1, aflatoxin B1; MMDA, multicomponent mycotoxin detoxifying agent.
[1]kg feed per kg egg mass.

fed corresponding mycotoxin levels without MMDA (T3). AFB1 and T2-toxin at the maximum tolerated dose level (T5) did significantly reduce overall feed intake (-3.7%), egg production (-9.7%), and overall egg mass (-9.5%) in comparison with the control group. With presence of MMDA at 2 g /kg feed (T6) and 3 g/kg feed (T7) the negative responses of mycotoxins at the maximum tolerated dosage were slightly reduced (overall feed intake: +0.8%; overall egg production: +1.5%; overall egg mass: +1.6%; overall FCR: -1%). Moreover, results indicated that MMDA in combination with mycotoxins at the guidance level seemed to be more efficient than those at the maximum tolerated dosage. There were no consistent treatment effects in overall body weight change, mean egg weight, and overall number of broken or dirty eggs. Comparisons among the

Table 2. Effects of MMDA on laying performance of hens from d 29 to d 42 on trial (d 260 to d 273 of age).

Treatment groups		T1	T2	T3	T4	T4	T6	T7	Oneway ANOVA
Hens	n°	15	15	15	15	15	15	15	*P*
Replicates	n°	5	5	5	5	5	5	5	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
d 29 to d 35 on trial (d 260 to d 266 of age)									
Body weight - end	g	2002.0 ± 68.6	1968.2 ± 69.9	2007.2 ± 57.3	1978.0 ± 55.1	1954.4 ± 62.3	1986.4 ± 78.6	1984.6 ± 62.6	0.876
Body weight change	g	5.8 ± 20.0	4.6 ± 31.5	14.6 ± 15.2	-0.8 ± 17.4	9.4 ± 22.4	19.0 ± 11.9	18.8 ± 20.4	0.674
Feed intake	g	856.8 ± 28.3	865.2 ± 12.7	836.5 ± 18.5	843.4 ± 12.3	830.2 ± 17.6	835.5 ± 22.9	833.4 ± 24.2	0.184
Egg production	n°	7.0 ± 0.2^{a}	6.8 ± 0.2a	6.3 ± 0.3ab	6.3 ± 0.5ab	5.7 ± 0.2b	5.8 ± 0.6b	5.8 ± 0.4b	**<0.001**
Egg weight	g	65.5 ± 1.7	65.5 ± 1.3	64.8 ± 2.0	65.8 ± 0.9	65.2 ± 1.1	65.8 ± 2.8	66.1 ± 0.3	0.916
Egg mass	g	458.2 ± 14.2^{a}	445.3 ± 7.2^{a}	410.7 ± 28.9^{ab}	416.5 ± 26.3^{ab}	369.8 ± 19.2^{b}	381.3 ± 36.3^{b}	383.1 ± 24.8^{b}	**<0.001**
Feed conversion ratio[1]		1.871 ± 0.084^{a}	1.943 ± 0.052^{ab}	2.046 ± 0.165^{abc}	2.033 ± 0.154^{abc}	2.252 ± 0.157^{c}	2.206 ± 0.203^{bc}	2.184 ± 0.177^{bc}	**0.003**
Broken eggs	n°	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0 ± 0	0.2 ± 0.4	0.984
Dirty eggs	n°	0.4 ± 0.5	0.4 ± 0.9	0.4 ± 0.9	0.2 ± 0.4	0.4 ± 0.9	0.4 ± 0.5	0 ± 0	0.946
d 36 to d 42 on trial (d 267 to d 273 of age)									
Body weight end	g	1998.4 ± 52.4	1990.0 ± 75.4	2000.2 ± 42.0	1991.2 ± 30.4	1971.8 ± 40.8	2001.6 ± 89.2	1981.2 ± 49.4	0.980
Body weight change	g	-3.6 ± 24.6	21.8 ± 14.9	-7.0 ± 20.8	13.2 ± 27.9	17.4 ± 22.6	15.2 ± 11.9	-3.4 ± 23.6	0.208
Feed intake	g	886.1 ± 18.9	882.0 ± 20.4	833.9 ± 33.3	847.0 ± 19.7	834.4 ± 48.1	831.6 ± 44.1	833.0 ± 11.1	0.136
Egg production	n°	6.7 ± 0.5^{b}	6.7 ± 0.2^{b}	6.0 ± 0.3^{ab}	6.1 ± 0.2^{ab}	6.0 ± 0.5^{ab}	5.7 ± 0.3^{a}	5.9 ± 0.3^{a}	**0.001**
Egg weight	g	66.1 ± 1.3	66.4 ± 0.9	66.3 ± 1.7	65.9 ± 1.0	66.4 ± 0.6	65.7 ± 1.4	65.4 ± 1.2	0.820
Egg mass	g	440.5 ± 29.5^{bc}	442.9 ± 14.9^{c}	397.4 ± 18.2^{ab}	404.5 ± 16.0^{abc}	398.1 ± 33.7^{ab}	376.7 ± 12.7^{a}	383.6 ± 17.0^{a}	**<0.001**
Feed conversion ratio[1]		2.017 ± 0.097	1.993 ± 0.063	2.100 ± 0.080	2.098 ± 0.130	2.114 ± 0.273	2.210 ± 0.143	2.176 ± 0.122	0.231
Broken eggs	n°	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0 ± 0	0 ± 0	0.914
Dirty eggs	n°	0.4 ± 0.9	0.4 ± 0.5	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.4 ± 0.5	0.2 ± 0.4	0.980

Abbreviations: AFB1, aflatoxin B1; MMDA, multicomponent mycotoxin detoxifying agent.
Different superscripts in same row are significant (a/b: $P \leq 0.05$).
[1]kg feed per kg egg mass.

Table 3. Effects of MMDA on overall laying performance of hens from d 1 to d 42 on trial (d 232 to d 273 of age).

Treatment groups		T1	T2	T3	T4	T4	T6	T7	Oneway ANOVA
Hens	n°	15	15	15	15	15	15	15	*P*
Replicates	n°	5	5	5	5	5	5	5	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
		d 1 to d 42 on trial (d 232 to d 273of age)							
Body weight start		1947.2 ± 87.1	1946.0 ± 147.6	1945.4 ± 37.7	1946.8 ± 44.7	1946.2 ± 67.6	1947.4 ± 41.9	1951.6 ± 44.2	1.000
Body weight - end	g	1998.4 ± 52.4	1990.0 ± 75.4	2000.2 ± 42.0	1991.2 ± 30.4	1971.8 ± 40.8	2001.6 ± 89.2	1981.2 ± 49.4	0.980
Body weight change	g	51.2 ± 91.4	44.0 ± 75.5	54.8 ± 24.4	44.4 ± 23.2	25.6 ± 46.2	54.2 ± 63.6	29.6 ± 54.7	0.975
Cumulative feed intake	g	5151.5 ± 73.0^{a}	5133.6 ± 102.5^{a}	5003.6 ± 60.7^{ab}	5042.5 ± 81.2^{ab}	4965.1 ± 91.0^{b}	4999.3 ± 47.6^{ab}	4997.8 ± 85.5^{ab}	**0.004**
Daily feed intake	g	122.7 ± 1.7^{a}	122.2 ± 2.4^{a}	119.1 ± 1.4^{ab}	120.1 ± 1.9^{ab}	118.2 ± 2.2^{b}	119.0 ± 1.1^{ab}	119.0 ± 2.0^{ab}	**0.004**
Cumulative egg production	n°	40.4 ± 0.^{8c}	40.2 ± 0.9^{bc}	37.3 ± 1.1^{a}	38.1 ± 0.6^{ab}	36.5 ± 1.3^{a}	36.7 ± 1.3^{a}	36.9 ± 1.4^{a}	**<0.001**
Mean egg weight	g	64.1 ± 0.6	64.7 ± 0.6	64.6 ± 0.7	64.5 ± 0.5	64.3 ± 0.6	64.5 ± 0.8	64.8 ± 0.6	0.636
Cumulative egg mass	g	2590.3 ± 42.8^{bc}	2603.0 ± 65.9^{c}	2413.2 ± 83.2^{a}	2458.7 ± 48.2^{ab}	2343.5 ± 68.1^{a}	2363.0 ± 61.9^{a}	2386.6 ± 83.8^{a}	**<0.001**
Cumulative feed conversion ratio[1]		1.989 ± 0.025^{ab}	1.974 ± 0.082^{a}	2.075 ± 0.075^{ab}	2.051 ± 0.052^{ab}	2.121 ± 0.092^{ab}	2.117 ± 0.073^{b}	2.097 ± 0.105^{ab}	**0.020**
Cumulative broken eggs	n°	1.8 ± 1.1	0.6 ± 0.9	1.0 ± 1.2	1.2 ± 0.8	1.2 ± 1.3	1.0 ± 0.7	0.8 ± 0.4	0.602
Cumulative dirty eggs	n°	2.0 ± 1.9	1.8 ± 1.3	1.6 ± 0.9	1.6 ± 1.1	1.6 ± 1.1	2.0 ± 0.7	1.0 ± 1.0	0.871

Abbreviations: AFB1, aflatoxin B1; MMDA, multicomponent mycotoxin detoxifying agent.
Different superscripts in same row are significant (a/b: $P \leq 0.05$).
[1]kg feed per kg egg mass.

weekly feeding phases revealed that negative responses of mycotoxin application at 2 dose levels increased with from the start of the second week onwards (Tables 1–3). Treatment of feed containing 70 μg AFB1/kg with fermentation liquor of *B. subtilis* ANSB060 has protective effect on eggshell quality and liver damage in layer birds (Salem et al., 2018). White Leghorn layer breeder hens provided with 10 μg AFB1/kg produced by *Aspergillus flavus* with 0.1 mg/kg vitamin E in feed has produced partial protective effect on egg quality and hatchability (Khan et al., 2014).

Blood Profiles

The hens fed diets containing AFB1 at 0.05 mg/kg feed in combination with T2-toxin at 1.5 mg/kg feed (T3) without inclusion of MMDA resulted in slightly or significantly enhanced means of leukocytes, hemoglobin (significant), mean corpuscular hemoglobin (**MCH**), MCH concentration (**MCHC**, significant), triglycerides and creatine as compared with the control group. The addition of MMDA at 3 g/kg feed to contaminated diets at the maximum tolerated dose level (T7) seemed to be slightly effective to counteract some of the mycotoxin promoted effects (leukocytes, calcium, cholesterol, triglycerides, creatine, glucose, albumins, globulins, and total protein) in comparison with the high mycotoxin level alone. In presence of MMDA at 2 g/kg feed (T6) changes in comparison with the high mycotoxin level alone were lower than those recorded for MMDA at 3 g/kg feed. Comparison between hens fed mycotoxins at the guidance level without or with MMDA at 1 g/kg feed showed slightly higher (potassium, aspartate-amino-transferase [**AST**], total cholesterol, glucose, globulins, and total protein) or reduced concentrations (hemoglobin, MCH, MCHC, calcium, triglycerides) as compared to contaminated hens fed mycotoxins at the guidance level(Tables 4–6). Alkaline phosphatase and AST are important liver enzymes related to protein metabolism and cell integrity (Walzem et al., 1993; Jiang et al., 2014). The poultry blood is found to exhibit elevated levels of ALT and AST after administration of AFB1 in diet as a result of liver damage(Han et al., 2008; Diaz et al., 2009; He et al., 2013; Gómez-Espinosa et al., 2017).The plasma of the birds provided with a diet containing AFB1 show declined content of cholesterol, triglycerides, total protein, albumin and globulin (Bailey et al., 2006; Siloto et al., 2013; Gholami-Ahangaran et al., 2016; Salem et al., 2018).

Pathological Examination

Results of pathological signs are presented in Tables 6 and 7 show the effect of increasing mycotoxins in feed on pathological examination at d 42 on trial (d 273 of age). Pathological alterations of liver, kidneys, gall bladder and bile duct in hens fed AFB1 and T2-toxin via spiked maize at the guidance or maximum tolerated dosage without MMDA application increased with increasing dose level of mycotoxins as compared with the control group. Grossly, there was a pale or yellowish discoloration of liver along with its size and distension of gall bladder and swollen kidneys bulging out of their sockets with congestion. However, fatty liver syndrome was also observed in hens fed diets without spiked maize. The addition of MMDA at dosage 1 to 3 g/kg feed did slightly modify pathological alterations of liver and kidneys in comparison with hens fed the contaminated diets at both dose levels without inclusion of MMDA. The greatest response was observed with feeding MMDA at

Table 4. Effects of MMDA on hematological traits at d 42 on trial (d 273 of age).

Treatment groups		T1	T2	T3	T4	T4	T6	T7	
Hens	n°	10	10	10	10	10	10	10	
Replicates	n°	10	10	10	10	10	10	10	
MMDA	g/kg		2		1		2	3	ANOVA
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
					Body weight				
Body weight	g								
					Hematological traits				
Erythrocytes	T/L	2.29 ± 0.04^{ab}	2.13 ± 0.12^{a}	2.30 ± 0.08^{ab}	2.19 ± 0.12^{ab}	2.28 ± 0.04^{ab}	2.32 ± 0.14^{b}	2.23 ± 0.16^{ab}	**0.021**
Leukocytes	G/L	7.71 ± 1.89	7.94 ± 1.76	8.77 ± 5.66	8.97 ± 1.23	7.01 ± 1.03	6.89 ± 1.45	7.14 ± 1.56	0.610
Lymphocytes	%	42.0 ± 6.6	44.9 ± 6.4	44.1 ± 7.7	47.0 ± 6.1	37.6 ± 6.7	36.6 ± 6.6	39.0 ± 8.5	0.172
Heterophils	%	55.3 ± 6.8	51.4 ± 6.6	51.3 ± 10.2	49.6 ± 5.9	58.2 ± 6.9	58.7 ± 6.4	56.7 ± 8.5	0.122
Monocytes	%	1.4 ± 0.8	2.6 ± 1.1	3.1 ± 2.6	1.7 ± 1.1	1.6 ± 1.5	1.7 ± 1.1	1.9 ± 0.4	0.253
Eosinophils	%	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0.8	0.8 ± 1.1	0.9 ± 1.2	0.6 ± 0.8	0.119
Basophils	%	1.3 ± 0.8	1.1 ± 1.1	1.4 ± 1.1	1.7 ± 1.1	1.8 ± 1.1	2.1 ± 1.3	1.9 ± 1.2	0.659
Hemoglobin	g/L	72.14 ± 3.44^{a}	74.43 ± 3.64^{a}	83.14 ± 7.84^{bc}	73.57 ± 10.72^{ab}	85.00 ± 1.91^{c}	86.14 ± 4.49^{c}	84.86 ± 4.38^{c}	**<0.001**
Hematocrit	L/L	0.23 ± 0.01^{b}	0.21 ± 0.01^{a}	0.22 ± 0.01^{ab}	0.22 ± 0.01^{ab}	0.22 ± 0.01^{ab}	0.22 ± 0.01^{ab}	0.21 ± 0.02^{ab}	**0.025**
MCV[1]	Fl	99.41 ± 2.31^{c}	96.54 ± 1.15^{abc}	94.47 ± 2.09^{ab}	97.66 ± 2.22^{bc}	94.63 ± 2.42^{ab}	93.70 ± 2.10^{a}	94.17 ± 2.77^{ab}	**<0.001**
MCH[2]	Pg	31.43 ± 0.33^{abc}	31.32 ± 0.26^{ab}	38.39 ± 2.92^{bcd}	34.01 ± 4.03^{a}	39.37 ± 0.77^{cd}	39.66 ± 1.06^{cd}	40.49 ± 1.42^{d}	**<0.001**
MCHC[3]	g/dl	31.61 ± 0.32^{a}	31.33 ± 0.52^{a}	36.29 ± 3.07^{b}	29.21 ± 9.36^{a}	37.24 ± 0.62^{b}	39.66 ± 1.06^{b}	40.49 ± 1.42^{b}	**<0.001**

Abbreviations: AFB1, aflatoxin B1; MCH, mean corpuscular hemoglobin, MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MMDA, multicomponent mycotoxin detoxifying agent.

Different superscripts in same row are significant (a/b: $P \leq 0.05$).

[1]Mean corpuscular volume;

[2]Mean corpuscular hemoglobin;

[3]Mean corpuscular hemoglobin concentration.

3 g/kg feed. Furthermore, data indicated no treatment effects on intestinal and respiratory tract, heart, and bursa of *Fabricius* (Tables 7 and 8). The administration of 10^8 cfu of *Berevibacillus laterosporus*/mL of drinking water declined necrosis in liver, enhanced production of proteins and antibodies and increased growth rate in quails provide with 2,500 μg of AFB1/kg produced by *A. parasiticus* (PTCC 5286) (Bagherzadeh Kasmani et al., 2012).

Effect on Organ and Tissue Weights

In Table 9 of dietary treatments on organ weights at d 42 of on trial (d 273 of age) are summarized. Homogeneity of variances was asserted using ANOVA which showed that equal variances could be assumed. Measured values among treatment groups were normally distributed (ANOVA). Feeding mycotoxins at the guidance dosage alone (T3) caused significantly

Table 5. Effects of MMDA on plasma concentrations of electrolytes and enzymes at d 42 on trial (d 273 of age).

Treatment groups		T1	T2	T3	T4	T4	T6	T7	
Hens	n°	10	10	10	10	10	10	10	
Replicates	n°	10	10	10	10	10	10	10	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
					Body weight				
Body weight	g								
					Electrolytes and enzymes				
Sodium	mmol/L	150 ± 4	153 ± 3	153 ± 2	152 ± 4	150 ± 1	151 ± 3	153 ± 1	0.209
Potassium	mmol/L	4.2 ± 0.7^{a}	4.6 ± 0.5^{ab}	4.4 ± 0.6^{ab}	5.4 ± 0.9^{ab}	4.8 ± 1.2^{ab}	5.8 ± 1.2^{ab}	5.6 ± 1.3^{b}	**0.015**
Chloride	mmol/L	113 ± 3^{x}	116 ± 3^{xy}	117 ± 1^{xy}	115 ± 3^{xy}	115 ± 1^{xy}	114 ± 3^{xy}	116 ± 2^{y}	*0.096*
Calcium	mmol/L	6.85 ± 0.50^{a}	6.72 ± 0.92^{a}	6.80 ± 0.56^{a}	4.67 ± 2.85^{ab}	4.86 ± 0.73^{ab}	4.71 ± 0.81^{b}	5.32 ± 1.13^{b}	**0.002**
Magnesium	mmol/L	0.99 ± 0.10	0.97 ± 0.05	0.94 ± 0.06	0.94 ± 0.09	0.95 ± 0.05	0.95 ± 0.06	0.95 ± 0.06	0.919
Phosphate	mmol/L	2.12 ± 0.43	2.29 ± 0.42	2.13 ± 0.22	1.97 ± 0.47	2.03 ± 0.45	1.89 ± 0.27	1.96 ± 0.47	0.583
AST[1]	U/L	216 ± 32	201 ± 20	194 ± 21	246 ± 73	220 ± 23	206 ± 19	207 ± 13	0.151
ALT[2]	U/L	<3	<3	<3	<3	<3	<3	<3	
GLDH[3]	U/L	7 ± 10	9 ± 11	9 ± 8	10 ± 3	9 ± 14	10 ± 11	9 ± 19	1.000
ALP[4]	U/L	647 ± 101	507 ± 117	590 ± 281	618 ± 285	656 ± 213	614 ± 175	668 ± 260	0.842

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; GLDH, glutamate dehydrogenase.

Different superscripts in same row are significant or trending (a/b: $P \leq 0.05$; x/y $0.05 < P \leq 0.10$).

[1]Aspartate transaminase.

[2]Alanine aminotransferase.

[3]Glutamate dehydrogenase.

[4]Alkaline phosphatase.

Table 6. Effects of MMDA on biochemical parameters in blood at d 42 on trial (d 273 of age).

Treatment groups		T1	T2	T3	T4	T4	T6	T7	
Hens	n°	10	10	10	10	10	10	10	
Replicates	n°	10	10	10	10	10	10	10	
MMDA	g/kg		2		1		2	3	ANOVA
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
					Body weight				
Body weight (g)	g								
					Biochemical parameters				
Total cholesterol	mmol/L	2.80 ± 0.40^{ab}	2.64 ± 0.77^{ab}	2.70 ± 0.27^{ab}	3.27 ± 0.76^{b}	1.85 ± 0.34^{a}	2.22 ± 0.76^{ab}	2.38 ± 0.77^{ab}	**0.005**
Triglycerides	mmol/L	14.35 ± 3.35	14.29 ± 4.48	15.01 ± 2.32	13.31 ± 9.25	8.29 ± 2.24	10.59 ± 5.58	12.22 ± 4.76	0.178
Urea	mmol/L	<0.80	<0.80	<0.80	<0.80	<0.80	<0.80	<0.80	
Total bilirubin	μmol/L	2.29 ± 0.30^{a}	2.01 ± 0.34^{ab}	1.79 ± 0.30^{b}	2.00 ± 0.40^{ab}	1.93 ± 0.28^{ab}	1.98 ± 0.19^{ab}	2.21 ± 0.11^{ab}	**0.043**
Creatine	μmol/L	9.24 ± 0.85	9.27 ± 1.61	11.66 ± 2.99	11.81 ± 3.62	12.16 ± 2.85	12.41 ± 5.29	11.11 ± 2.63	0.302
Glucose	mmol/L	7.29 ± 2.20	6.64 ± 1.56	5.52 ± 1.51	9.02 ± 3.64	8.59 ± 2.20	9.28 ± 3.78	$8.22 \pm 1\ 2.72$	0.109
Albumins	mmol/L	17.0 ± 0.2^{x}	16.2 ± 1.0^{xy}	16.8 ± 1.0^{xy}	16.8 ± 0.8^{xy}	14.8 ± 0.7^{y}	15.3 ± 2.7^{xy}	15.8 ± 1.9^{y}	*0.060*
Globulins	mmol/L	33.0 ± 1.8^{b}	28.9 ± 2.5^{ab}	29.4 ± 2.9^{ab}	32.1 ± 4.7^{b}	25.9 ± 3.0^{a}	25.6 ± 4.4^{a}	26.1 ± 3.5^{a}	**<0.001**
Total protein	mmol/L	50.0 ± 1.8^{c}	45.2 ± 3.4^{abc}	46.1 ± 3.8^{abc}	48.9 ± 4.0^{bc}	40.7 ± 3.5^{a}	40.8 ± 6.8^{a}	41.9 ± 5.3^{b}	**<0.001**

Different superscripts in same row are significant or trending (a/b: $P \leq 0.05$; x/y $0.05 < P \leq 0.10$).

Table 7. Effect of MMDA on pathological examination of intestinal and respiratory tract in laying hens at d 42 on trial (d 27 age).

Treatment groups		T1	T2	T3	T4	T5	T6	T7	*P* ANOVA
Hens	n°	10	10	10	10	10	10	10	
Replicates	n°	10	10	10	10	10	10	10	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
					Body weight				
Body weight	g	2066.3 ± 108.6^{a}	2050.1 ± 115.7^{ab}	2012.4 ± 62.6^{ab}	2013.6 ± 66.8^{ab}	1945.4 ± 56.3^{b}	1987.6 ± 83.2^{ab}	1980.0 ± 41.0^{ab}	**0.023**
					Intestinal tract				
Larynx, oesophagus, crop, proventriculus/ gizzard, small intestine, large intestine, caeca, cloaca					No pathological signs				
					Respiratory tract				
Trachea, bronchi, lungs					No pathological signs				

Different superscripts in same row are significant (a/b: $P \leq 0.001$).

enhanced relative weights of kidneys (+13.8%) and slightly increased relative weights of liver in comparison with the control group (+8.5%). AFB1 and T2-toxin at the maximum tolerated dosage (T5) did significantly increase relative weights of liver, kidneys and spleen as compared with the control group (liver: +8.5%; kidneys: +22.4%; spleen: +9.1%). Results responded linearly to increasing dietary AFB1 and T2-toxin levels, but significant effect of dose level was only found regarding relative weight of kidneys (+7.6%). Simultaneous presence of MMDA in diets containing AFB1 and T2-toxin at both dose levels did on average slightly reduce relative organ weights in comparison to contaminated diets alone (liver: -2.1%; kidneys: -1.4%; spleen: -8.3%); effect of MMDA dose level was not evident. No treatment response on relative breast weight was observed, nor in dry matter content of organs and breast muscle. AFB1 is associated with liver damage through the suppression of the activity of anti-inflammatory cytokines and antioxidative enzymes, increased apoptotic activity (Liao et al., 2014; Ma et al., 2015; Muhammad et al., 2018; Wang et al., 2019) and deposition of lipid in hepatocytes (Tejada-Castañeda et al., 2008; Siloto et al., 2013).

An inclusion of 5 g of hydrated calcium aluminosilicate or 5.0 to 7.5 g of bentonite, 15 g of clinoptilolite in the diet of poultry birds containing 2,000 to 2,500 μg of AFB1 produced by *A. parasiticus* reduced the detrimental effect on performance (Chen et al., 2014; Dos Anjos et al., 2015; Shannon et al., 2017) and liver by declining the concentration of AFB1 from 8.3 to 1.5 μg/kg (Neeff et al., 2013). Studies have reflected the renal enlargement after administration of birds with AFB1 (Şehu et al., 2005; Uyar et al., 2016; Gómez-Espinosa et al., 2017) and may be attributed to tubular distension, increased thickness of glomerular basement membrane and mesangial cells (Karaman et al., 2005; Liang et al., 2015). Introduction of *B. subtilis* (ANSB060) (organic binder) at the dose of 2.0 g/kg in the diet of broiler birds containing 70 μg AFB1/kg has enhanced the FCR and declined the level of accumulation of AFB1 from 0.24 to 0.09 μg/kg in liver and 7 to 1.5 μg/kg in intestine (Fan et al., 2013; Fan et al., 2015). The relative weight of liver in the broiler birds was within normal range fed with 5.0 g of hydrated sodium aluminosilicate/kg of feed containing 2,000 μg AFB1/kg obtained from *Aspergillus* (Chen et al., 2014).

Table 8. Effect of MMDA on pathological examination of organs in laying hens at d 42 on trial (d 273 of age).

Treatment groups		T1	T2	T3	T4	T5	T6	T7	*P* ANOVA
Total birds per group	n°	10	10	10	10	10	10	10	
Repetitions	n°	10	10	10	10	10	10	10	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
					Body weight				
Body weight	g	2066.3 ± 108.6[a]	2050.1 ± 115.7[ab]	2012.4 ± 62.6[ab]	2013.6 ± 66.8[ab]	1945.4 ± 56.3[b]	1987.6 ± 83.2[ab]	1980.0 ± 41.0[ab]	**0.023**
					Organs				
Liver		Enlargement, yellowish, rounded borders, fatty: n = 2 Sporadic petechial hemorrhages n= 1 No pathological signs: n = 8	Enlargement, yellowish, rounded borders, fatty: n = 3 Sporadic petechial hemorrhages n= 1 No pathological signs: n = 7	Enlargement, yellowish, rounded borders, fatty: n = 4 Sporadic petechial hemorrhages n= 1 No pathological signs: n = 5	Enlargement, yellowish, rounded borders, fatty: n = 5 Sporadic petechial hemorrhages n= 2 No pathological signs: n = 3	Enlargement, yellowish, rounded borders, fatty: n = 4 Sporadic petechial hemorrhages n= 1 No pathological signs: n = 5	Enlargement, yellowish, rounded borders, fatty: n = 5 Sporadic petechial hemorrhages n= 2 No pathological signs: n = 3	Enlargement, yellowish, rounded borders, fatty: n = 3 Sporadic petechial hemorrhages n= 1 No pathological signs: n = 6	
Gall bladder		Distension: n = 2 No pathological signs: n = 8	Distension: n = 3 No pathological signs: n = 7	Distension: n = 3 No pathological signs: n = 7	Distension: n = 4 No pathological signs: n = 6	Distension: n = 5 No pathological signs: n = 5	Distension: n = 3 No pathological signs: n = 7	Distension: n = 4 No pathological signs: n = 6	
Bile duct		No pathological signs	No pathological signs	Distension: n = 4 No pathological signs: n = 6	Distension: n = 2 No pathological signs: n = 8	Distension: n = 3 No pathological signs: n = 7	Distension: n = 2 No pathological signs: n = 8	Distension: n = 1 No pathological signs: n = 9	
Spleen		No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	
Kidneys		No pathological signs	No pathological signs	Enlargement and pale: n = 3 Sporadic petechial hemorrhages n = 1 No pathological signs: n = 6	Enlargement and pale: n= 2 No pathological signs: n = 8	Enlargement and pale: n = 4 Sporadic petechial hemorrhages n= 1 No pathological signs: n = 5	Enlargement and pale: n= 4 No pathological signs: n = 6	Enlargement and pale: n = 3 No pathological signs: n = 7	
Bursa of *Fabricius*		No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	
Heart		No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	No pathological signs	

Different superscripts in same row are significant (a/b: $P \leq 0.001$).

Table 9. Effects of MMDA on body weight, liver weight, spleen weight, kidney weight, and breast muscle weight of laying hens at d 42 on trial (d 273 of age).

Treatment groups		T1	T2	T3	T4	T5	T6	T7	*P* value
Total birds per group	n°	10	10	10	10	10	10	10	
Repetitions	n°	10	10	10	10	10	10	10	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
Body weight at d 42 on trial (d 273 of age)									
Body weight	g	2066.3 ± 108.6^{a}	2050.1 ± 115.7^{ab}	2012.4 ± 62.6^{ab}	2013.6 ± 66.8^{ab}	1945.4 ± 56.3^{b}	1987.6 ± 83.2^{ab}	1980.0 ± 41.0^{ab}	**0.023**
Liver at d 42 on trial (d 273 of age)									
Weight (as is)	g	41.07 ± 2.57^{a}	41.45 ± 3.28^{ab}	43.43 ± 1.73^{abc}	43.60 ± 2.25^{abc}	45.36 ± 2.41^{c}	45.32 ± 4.06^{c}	45.04 ± 2.65^{bc}	**<0.001**
Relative weight (as is)	% of BW	1.99 ± 0.06^{a}	2.02 ± 0.13^{ab}	2.16 ± 0.12^{abc}	2.16 ± 0.07^{bc}	2.33 ± 0.11^{c}	2.28 ± 0.21^{c}	2.28 ± 0.15^{c}	**<0.001**
Dry matter	%	28.37 ± 1.18^{a}	28.44 ± 0.87^{ab}	28.71 ± 0.73^{abc}	28.07 ± 1.11^{a}	28.93 ± 1.16^{abc}	30.10 ± 1.17^{c}	29.90 ± 1.41^{bc}	**<0.001**
Kidneys at d 42 on trial (d 273 of age)									
Weight (as is)	g	12.00 ± 0.63^{a}	12.18 ± 0.59^{b}	13.32 ± 0.77^{c}	13.49 ± 0.41^{c}	13.80 ± 0.34^{c}	14.05 ± 0.60^{c}	13.95 ± 0.34c	**<0.001**
Relative weight (as is)	% of BW	0.58 ± 0.02^{a}	0.59 ± 0.03^{a}	0.66 ± 0.03^{b}	0.67 ± 0.01^{b}	0.71 ± 0.01^{c}	0.70 ± 0.02^{c}	0.70 ± 0.02^{c}	**<0.001**
Dry matter	%	20.66 ± 1.34	20.73 ± 1.63	20.94 ± 1.21	20.77 ± 1.12	20.01 ± 1.93	20.12 ± 1.19	20.38 ± 1.03	0.685
Spleen at d 42 on trial (d 273 of age)									
Weight (as is)	g	2.29 ± 0.20^{c}	2.30 ± 0.14^{c}	1.97 ± 0.13^{a}	2.23 ± 0.23^{abc}	2.40 ± 0.23^{c}	2.26 ± 0.24^{bc}	2.02 ± 0.16^{ab}	**<0.001**
Relative weight (as is)	% of BW	0.11 ± 0.01^{abc}	0.11 ± 0.01^{bc}	0.10 ± 0.01^{a}	0.11 ± 0.01^{abc}	0.12 ± 0.01^{c}	0.11 ± 0.01^{bc}	0.10 ± 0.01^{ab}	**<0.001**
Dry matter	%	71.76 ± 1.74	71.14 ± 1.32	70.96 ± 0.97	71.19 ± 1.64	70.81 ± 3.20	70.36 ± 2.32	70.67 ± 1.76	0.795
Breast at d 42 on trial (d 273 of age)									
Weight (as is)	g	87.31 ± 4.21^{bc}	88.31 ± 4.58^{c}	85.30 ± 2.91^{abc}	86.15 ± 1.36^{abc}	82.82 ± 2.28^{a}	83.79 ± 2.95^{ab}	83.89 ± 2.75^{ab}	**0.004**
Relative weight (as is)	% of BW	4.23 ± 0.13	4.31 ± 0.14	4.24 ± 0.15	4.28 ± 0.17	4.26 ± 0.11	4.22 ± 0.18	4.24 ± 0.13	0.801
Dry matter	%	28.07 ± 1.30	27.19 ± 1.95	27.81 ± 2.05	28.10 ± 2.27	26.69 ± 0.81	27.52 ± 1.91	26.55 ± 1.73	0.289

Different superscripts in same row are significant (a/b: $P \leq 0.05$).

Egg Characteristics

Results of egg characteristics determined at d 40 and d 42 on trial (d 271 of age, d 273 of age are given in Table 10. Significant treatment effects were identified on eggshell weight. Means of hens fed diets containing AFB1 and T2-toxin at 2 dose levels each without inclusion of MMDA (T3, T5) were significantly decreased by an average of 6.2% as compared with the control group. Application of MMDA to mycotoxin contaminated diets at 2 dose levels each (T4, T6, T7) did not markedly modify eggshell weight in comparison to hens fed mycotoxin contaminated diets alone. Moreover, inclusion of MMDA at 2 g/kg feed to uncontaminated diet (T2) did slightly reduce eggshell weight as compared with the control group (-3.1%). Nevertheless, eggshell stability was nearly similar across treatment groups. Although egg yolk color among treatment groups was not significantly different, mycotoxin contamination did slightly decrease egg yolk color as compared with the control group. In presence of MMDA mycotoxin effects on egg yolk color seemed to be slightly diminished. A marked reduction in yolk color score and eggshell thickness was documented in a study after administration of layer hens with a feed containing 2,500 μg of AFB1/kg from 14 d to 280 d of age (Pandey and Chauhan, 2007).

Residue Levels in Tissues and Eggs

Deposition of AFB1 and T2-toxin and their main metabolites aflatoxin M1 and HT-2 toxin in selected tissues and eggs (without eggshell) are given in Table 11. Tissues and eggs of hens fed diets with naturally corn without or with MMDA were tested negative (under the limit of detection: = 0) for AFB1, T2-toxin and their

Table 10. Effects of MMDA on egg characteristics at d 40 and d 42 on trial (d 271 and d 273 of age).

Treatment groups		T1	T2	T3	T4	T5	T6	T7	*P* value
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
Eggshell stability & egg yolk color (d 40 on trial)									
Eggs	n°	14	13	14	13	14	13	11	
Egg weight (as is)	G	65.6 ± 1.1	65.1 ± 1.6	64.8 ± 0.9	65.1 ± 1.1	65.0 ± 0.8	65.1 ± 1.2	64.9 ± 1.0	0.645
Eggshell stability	N	62.3 ± 1.1	61.9 ± 1.9	61.7 ± 2.7	61.4 ± 1.8	61.8 ± 2.1	61.0 ± 1.6	61.1 ± 1.8	0.637
Egg yolk color fan 1)		13.5 ± 0.1^{x}	13.4 ± 0.3^{xy}	13.1 ± 0.4^{y}	13.4 ± 0.2^{xy}	13.2 ± 0.5^{xy}	13.2 ± 0.4^{xy}	13.3 ± 0.3^{xy}	*0.067*
Weight of eggshell, egg mass and their dry matter contents (d 42 on trial)									
Eggs	n°	10	10	10	10	10	10	10	
Egg weight (as is)	g	66.2 ± 1.5	65.8 ± 1.2	64.2 ± 2.0	65.0 ± 2.1	64.7 ± 1.0	65.2 ± 1.2	65.2 ± 2.1	0.173
Eggshell weight (as is)	g	6.5 ± 0.2^{a}	6.3 ± 0.2^{ab}	6.1 ± 0.2^{b}	6.2 ± 0.2^{ab}	6.1 ± 0.3^{b}	6.1 ± 0.2^{b}	6.1 ± 0.2^{b}	**0.001**
Dry matter of eggshell	%	88.85 ± 1.65	88.97 ± 0.72	88.55 ± 1.15	88.96 ± 1.28	89.02 ± 1.28	88.97 ± 0.77	89.08 ± 0.92	0.964
Egg mass without shell (as is)	g	59.7 ± 1.4	59.5 ± 1.1	58.1 ± 1.8	58.8 ± 2.0	58.6 ± 0.9	59.1 ± 1.1	59.0 ± 1.9	0.293
Dry matter of egg mass without shell	%	24.17 ± 0.47	24.16 ± 0.52	24.10 ± 0.37	24.08 ± 0.25	24.01 ± 0.26	23.98 ± 0.45	24.05 ± 0.36	0.916

Different superscripts in same row are significant or trending (a/b: $P \leq 0.05$; x/y $0.05 < P \leq 0.10$).

Table 11. Effects of MMDA on residue levels of mycotoxins and their main metabolites in tissues and eggs of laying hens at d 42 on trial (d 273 of age).

Treatment groups		T1	T2	T3	T4	T5	T6	T7	*P* value
Total birds per group	n°	10	10	10	10	10	10	10	
Repetitions	n°	10	10	10	10	10	10	10	
MMDA	g/kg		2		1		2	3	
AFB1	mg/kg			0.05	0.05	0.50	0.50	0.50	
T2-toxin	mg/kg			1.5	1.5	2.0	2.0	2.0	
					Liver at d 42 on trial (d 273 of age)				
AFB1	μg/kg DM	0^{a}	0^{a}	0.19 ± 0.06^{a}	0.16 ± 0.03^{a}	0.93 ± 0.24^{b}	0.89 ± 0.08^{a}	0.77 ± 0.10^{a}	**<0.001**
AFM1	μg/kg DM	0	0	0	0	0	0	0	1.000
T2-toxin	μg/kg DM	0	0	0	0	0	0	0	1.000
HT-2 toxin	μg/kg DM	0	0	0	0	0	0	0	1.000
					Kidneys at d 42 on trial (d 273 of age)				
AFB1	μg/kg DM	0^{a}	0^{a}	0.12 ± 0.02^{b}	0.12 ± 0.02^{b}	0.99 ± 0.15^{d}	0.70 ± 0.06^{c}	0.71 ± 0.07^{c}	**<0.001**
AFM1	μg/kg DM	0^{a}	0	0	0	0.05 ± 0.0	0	0	0.872
T2-toxin	μg/kg DM	0^{a}	0^{a}	0.05 ± 0.11^{a}	0^{a}	0.84 ± 0.90^{b}	0.09 ± 0.11^{a}	0^{a}	**<0.001**
HT-2 toxin	μg/kg DM	0^{a}	0^{a}	0.55 ± 0.90^{b}	0.62 ± 0.84^{b}	1.27 ± 1.35^{c}	0.22 ± 0.45^{b}	0^{a}	**<0.001**
					Breast at d 42 on trial (d 273 of age)				
AFB1	μg/kg DM	0	0	0	0	0	0	0	1.000
AFM1	μg/kg DM	0	0	0	0	0	0	0	1.000
T2-toxin	μg/kg DM	0	0	0	0	0	0	0	1.000
HT-2 toxin	μg/kg DM	0	0	0	0	0	0	0	1.000
					Egg yolk & egg albumen at d 42 on trial (d 273 of age)				
AFB1	μg/kg DM	0	0	0	0	0	0	0	1.000
AFM1	μg/kg DM	0	0	0	0	0	0	0	1.000
T2-toxin	μg/kg DM	0	0	0	0	0	0	0	1.000
HT-2 toxin	μg/kg DM	0	0	0	0	0	0	0	1.000

0 = Level under detection.
Different superscripts in same row are significant (a/b: $P \leq 0.05$).

metabolites (aflatoxin M1, HT-2 toxin). With increasing dosage of AFB1 and HT-2 toxin, residue levels of AFB1 in liver tissues increased significantly up 0.93 μg/kg DM as compared with the control group whereas deposition of T2-toxin, aflatoxin M1 and HT-2 toxin was not detected. In kidneys retention of AFB1, T2-toxin and their metabolites increased dose dependently, whereby modifications between the low and high contamination rate were significant. With application of graded dose levels of MMDA into the high contaminated diets both mycotoxins and their metabolites decreased significantly in comparison to the corresponding mycotoxin containing diets without using MMDA. Comparisons between both dose levels of MMDA indicated the greatest response at 3 g/kg feed. Moreover, at the low contamination level effects of MMDA on deposition of AFB1 and T2-toxin were neglectable. Breast and eggs were always tested negative for AFB1, T2-toxin and their main metabolites (aflatoxin M1, HT-2 toxin). The chickens fed with 40 μg AFB1/kg bw obtained from *A. flavus* and 3.0 g of hydrated sodium calcium aluminosilicate/kg has increased the excretion and reduced the accumulation of AFB1 in liver and decreased the relative liver weight (Liu et al., 2018).

CONCLUSIONS

AFB1 and T-2 exposure in hens resulted in decreased egg production, decreased eggshell weights, changes in blood parameters, and pathological alterations in liver and kidney tissues in a dose-dependent manner. The addition of MMDA to the diet reduced the changes in the aforementioned parameters as well as the buildup of metabolites in liver and kidney tissues, which indicated the specific binding to AFB1 and T-2 toxin. The current study suggests that MMDA can be utilized in laying hen feed to reduce the negative effects brought on by AFB1 and T-2 toxins.

MATERIALS AND METHODS

Animals, Identification, Standard Health Program and Husbandry

A total of 105 Lohmann Brown laying hens (age-233 d; weight-1,950 g) were purchased from a local commercial source. The bird exhibiting the signs of injury or disease was excluded from selection. At arrival selected hens were randomly assigned to 35 pens with bedding of *Giant Miscanthus* grass chopped into 1-inch pieces, within a climate-controlled barn for laying hens according to body weight and laying performance. Adult layers were acclimatized for 7 d and supplemented with control diet. Following acclimatization, pens were distributed to the different treatments such that there were 15 birds per treatment and 3 hens per pen (replicate). Pens were labeled with texts and colored codes (T1: white, T2: green; T3: yellow, T4: blue, T5: red; T6: grey; T7: brown) to identify treatments. Pens were measuring 2.2 × 2.0 m, providing 1.5 m^2 per bird. Feeder adjustment (which controls feed flow) was checked daily and adjusted as necessary to ensure that approximately 50% of the bottom of the feed trough was visible and 50% of the feed trough was covered with feed. This was minimizing spillage of feed. Any spoiled or wet feed was removed daily and collected in buckets. After weighing aliquots, waste feed was freeze-dried and amounts were

used for determination of the correct feed intake. Drinkers were checked daily to ensure adequate water flow.

Throughout the 7-d adaption period and the 42-d experimental period the poultry house was provided with controlled climate and forced ventilation (air speed about 0.5 m/s). The room temperature was kept at about 21.5°C throughout the 7-d adaption period and the following 42-d experimental period. The relative humidity was ranging between 55 and 60%. Hens were maintained on a 16 h light and an 8 h dark schedule per day with an average light intensity of about 365 lux throughout the experiment.

Measures to Avoid Cross-Contamination, Diet Composition, and Manufacture

All diets were manufactured in the institute mill (registration number: DE-BE-100001) that ensured that all stages of production, processing and distribution under their control were carried out in accordance with EU Community legislation, national law compatible therewith, and good practice. According to the REGULATION (EC) No 183/2005 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 12 January 2005 laying down requirements for feed hygiene; feed businesses was met several conditions relevant to their operations, concerning facilities, equipment, personnel, production, quality control, storage and documentation, in order to ensure both feed safety and product traceability. The application of hazard analysis and critical control points principles to the production of feed is the medium-term objective of European hygiene legislation. Moreover, the traceability of feed and feed ingredients throughout the feed chain is an essential element in ensuring feed safety. Regulation (EC) No 178/2002 contains rules to ensure the traceability of feed and feed ingredients and provides a procedure for the adoption of implementing rules applicable to specific sectors.

The basal ingredients for the diets were provided by the institute; MMDA containing modified zeolite (Clinoptilolite), *B. subtilis, B. Licheniformis, S. cerevisiae* cell wall and silymarin and premixtures of AFB1 and T2-toxin based on maize were supplied by PATENT CO DOO (https://global.patent-co.com/).The amounts of all the basal ingredients as well as MMDA and the mycotoxin containing premixes to produce the diets were recorded. The total amount of feed manufactured for the study was amounting to 800 kg. The batch was subsequently divided into 7 aliquots (T1: 200 kg; T2: 100 kg; T3: 100 kg; T4: 100 kg; T4: 100 kg; T6: 100 kg; T7: 100 kg) for mixing the experimental diets. Records of diet mixing were maintained. Diets were prepared without antibiotics, coccidiostats, antimicrobials, enzymes, or growth promoters (e.g., organic acids, probiotics, and other botanicals).

Dose levels of MMDA were supplemented at the expense of Tixosil (>97% silicon dioxide). In addition, AFB1 and T2-toxin via spiked maize was included to the experimental diets at the expense of uncontaminated maize. AFB1 mycotoxin was produced by infecting sterile corn with an in-house strain of Aspergillus mold, and T-2 by infecting either corn, or oat with *Fusarium langsethiae* Fe 2391strain. Water was added in the amount that measured 30% of the weight of the substrate for AFB1 and 20 to 30% for T-2. The Aspergillus infected corn was incubated at 25°C for 21 d. *F. langsethiae* infected corn was incubated for 15 d with a temperature regimen alternating between 15°C and 22.5°C. After the incubation period, the contaminated materials were dried in the drying oven at 105° for 3 d. The material was then left to cool, ground in the Perten mill and the mycotoxin content was measured with LC-MS/MS. For proper mixing premixtures (6 kg each) containing aliquots of basal diet and Tixosil & uncontaminated maize (T1), Tixosil & MMDA & uncontaminated maize (T2), Tixosil & uncontaminated/spiked maize (T3); Tixosil & MMDA & uncontaminated/spiked maize (T4), Tixosil & spiked maize (T5), Tixosil & MMDA & spiked maize (T6), and MMDA & spiked maize (T7) were produced by very gentle mixing and included to the corresponding batches of 194 kg (T1) and 94 kg each (T2−T7) under a minimum of dust formation prior mixing into the remaining feed at a minimum of dust formation. It is recommended that ventilation inside the mixing equipment was reduced to a minimum. To avoid contamination with previous productions, feed was manufactured in an appropriate rank order starting with the control diet and with a neutral meal mixing in between each of the diets. The rank of order was T1, T2, T3, T4, T5, T6, and T7, respectively. Amounts of all the basal ingredients and the required test product in form of premixes to produce the diets are recorded (Table 12) and the analysis of feed after mixing is shown in Table 13.

Table 12. Feed and test item required.

Treatment	AFB1 mg/kg	T2-Toxin mg/kg	MMDA g/kg	N° hens	Total feed/ treatment (kg)	AFB1 required Mg	T2-Toxin, requried mg	MMDA, required g
T1				15	200			
T2			2	15	100			200
T3	0.05	1.50		15	100	5.0	150	
T4	0.05	1.50	1	15	100	5.0	150	100
T5	0.50	2.00		15	100	50.0	200	
T6	0.50	2.00	2	15	100	50.0	200	200
T7	0.50	2.00	3	15	100	50.0	200	300
Totals				**105**	**800**	**160.0**	**900**	**800**

Table 13. Key proximate analysis and mycotoxin concentrations in the experimental diets (as fed).

Treatment groups		T1	T2	T3	T4	T5	T6	T7
Dry matter	g/kg	902.3	902.1	902.6	901.9	902.4	902.5	901.8
Crude protein	g/kg	168.5	168.7	168.5	169.1	168.8	168.6	169.2
Crude fiber	g/kg	27.5	27.2	27.5	27.8	27.3	27.7	27.3
Crude fat	g/kg	47.9	48.1	47.8	48.2	47.9	47.7	48.1
Crude ash	g/kg	125.5	126.8	125.7	128.2	125.8	128.5	130.4
Starch	g/kg	418.3	418.8	418.0	418.6	417.4	417.8	418.3
Sugars	g/kg	38.3	37.7	38.1	38.5	37.8	38.0	38.6
Calcium	g/kg	38.3	37.9	38.0	37.8	38.3	38.4	37.6
Phosphorus (total)	g/kg	5.6	5.8	5.7	5.6	5.9	5.6	5.9
Sodium	g/kg	1.8	1.8	1.8	1.9	1.8	1.8	1.9
AFB1	μg/kg	<0.10	<0.10	53.6	51.7	497.3	489.9	492.7
T2-toxin	μg/kg	29	27	1583	2012	1993	1972	1988
Ochratoxin	μg/kg	<1	<1	<1	<1	<1	<1	<1

Subsamples of each diet were collected during the run out of the feed from the feed mixer to the bagging unit. About 6 subsamples were obtained at a port in the auger line at equally spaced intervals between the beginning of run out and the end of run out. Subsamples were subsequently mixed. Representative samples of the mixture were obtained by splitting the samples from the mixer using a sample splitting device. Two samples (2 × 500 g) were taken for proximate and mycotoxin analysis and storage at the institute. Samples were labeled with the unique study code LH 5/20, the treatment code (T1, T2, T3, T4, T5, T6, and T7), the type of diet (layer), the date of manufacture and the analysis required (proximate, mycotoxins). Samples were stored in standard polyethylene bags and back up samples were frozen and stored at -18°C until further analysis.

Body Weight Measurement and Egg Characteristics

All hens were observed twice daily for any abnormalities, abnormal behavior, and clinical signs of sickness throughout the 42-d experimental period. This included the exterior (with special emphasis on skin, feathers, eyes, and visible mucous membranes), abnormal locomotion, movements and posture, reduced movement, presence of convulsions or paralysis, stereotypes, bizarre behavior as well as autonomous activities (e.g., abnormal breathing and body surface temperature) and morbidity/mortality. Any signs of abnormal behavior or a general change in feeding habits were recorded, if necessary. The following clinical sign key was used: individual body weights were recorded every week, and the feed supplied to each hen over each preceding week. Body weight change of hens was calculated by using the body weight at the end of each period minus the body weight at the start of each period. Feed consumption per hen was estimated as the total feed supplied per pen and period corrected for dispersed/leftover feed per pen. Eggs and their weights were recorded daily per hen and were summarized in weekly periods per hen. Feed conversion ratio (g feed/g egg) was calculated from the relationship of weekly corrected feed intake and egg mass for this period.

Blood Profile

Venous blood samples were taken at the end of the 42-d feeding period from 10 hens per treatment (2 hen per pen) selected for body weights closest to the average of the corresponding treatment group. Samples were collected from the *vena cutanea* into plain and heparinized or EDTA containing tubes. Samples were analyzed for hematological variables (erythrocytes, leukocytes, differential hemogram: lymphocytes, monocytes, eosinophils,

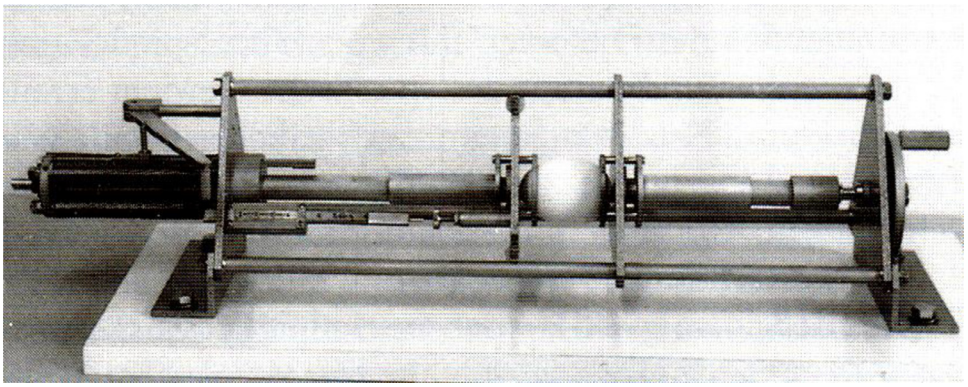

Figure 1. Apparatus for measuring the eggshell breaking strength.

basophils, neutrophils, hemoglobin, hematocrit, mean corpuscular volume (MCV), MCH, and MCH concentration (MCHC)) as well as for electrolytes (sodium, potassium, chlorine, calcium, and inorganic phosphate). Additionally, biochemical parameters (total cholesterol, triglycerides, bilirubin, urea, glucose, albumin, total protein) and enzyme activities (AST, gamma-glutamyltransferase, glutamate-dehydrogenase) were measured. Hens used for venous blood sampling before were killed by stunning and sacrificed by exsanguination for postmortem external and internal macroscopically examination by a veterinarian in accordance with the necropsy key.

Egg Shell Stability

All eggs collected at d 40 on trial were used for determination of eggshell stability and egg yolk color. Egg yolk color was evaluated by the Roche Yolk Color Fan (15 = dark orange; 1 = light pale). The eggshell stability was determined in an apparatus which compresses each egg between flat plates to measure breakage force (Figure 1). This procedure was carried out on eggs with the major axis parallel to the compression surfaces (force applied at equator). The figure of the equipment is shown above.

Mycotoxins and Metabolites Analysis in Organs and Eggs

Additionally, weights of liver, spleen, breast, and kidneys were monitored from each eviscerated carcass. Afterwards the liver, breast and kidneys were packed into polyethylene bags and kept at -20°C before being freeze-dried and subjected to chemical analyses. Ten eggs per treatment (2 eggs per pen) were collected at d 42 on trial for mycotoxin analysis. After weighing eggs without eggshell were packed into polyethylene bags and kept at -20°C before being freeze-dried and subjected to chemical analyses.

AFB1 and T-2 Toxin and Their Metabolites Analysis

The analysis of AFB1, B2, G1, G2, M1, T-2 and HT-2 toxins and their metabolites in the samples was performed using 6460c MS/MS QQQ with Jet Stream electrospray ion source, Agilent Technologies. The method was developed and validated in house (in communication for publication). The LOQ (μg/kg) for AFB1, AFB2, AFG1, AFG2 was 0.1 μg/kg, 0.2 μg/kg for T-2 and 1 μg/kg for HT-2 toxin. To compensate the matrix effect in electrospray ionization, as internal standard, isotopically labeled ($^{13}C_{24}$) AFB1 CRM BiopureTM—(0.5 μg/mL) for aflatoxins and ($^{13}C_{24}$) T-2 toxin; CRM BiopureTM—(25 μg/mL) for T-2/HT-2 toxins were used. The recovery of more than 75% was recorded for all the toxins. The method was linear from 0.1 to 1.2 μg/kg for aflatoxins, 0.2 to 4.0 μg/kg for T-2 and 1 to 20 μg/kg for HT-2 toxin.

The tissue samples were finely grounded and thoroughly mixed using a blender. A 2g test portion was removed for analysis. The samples were then extracted using a 10 mL extraction mixture (80% Acetonitrile: 15% Water: 5%Formic acid) and by shaking the mixture in an orbital shaker at 200 rpm for 1 h at room temperature. After extraction, this portion was centrifuged at 4 200 × g for 5 min, 7 mL of supernatant were removed and placed into another conical tube. The samples were cleaned by adding 2.8 g MgSO4 and 0.7g NaCl to the supernatant and vortexed for 60 s. These tubes were centrifuged at 4200 × g for 5 min. A 1 mL solution was removed from the supernatant and diluted with 250 μL water. Further clean-up was performed on Captiva EMR Lipid cartridge (Agilent): 1.25 mL supernatant were passed through the cartridge (by gravity) and collected into a 15 mL centrifuge tube. When all the extract has passed through the cartridge, 400 μL of the extraction solvent was added and collected into the same centrifuge tube. The extract in the evaporator (CHRIST RVC 2-18 CD plus) was evaporated at 1500 rpm under 40°C. Then, 500 μL solvent for reconstitution (50% Acetonitrile: 50% Water, containing 0.1% Formic acid) was add to the evaporated sample and vortexed well. The prepared samples were filtered across a nylon membrane syringe (pore size 0.22 μm) into a glass vial and vortexed. The samples were run on LC-MS/MS using analytical column Agilent ZORBAX Rapid Resolution HD 2.1*50mm 1.8μm and guard column ZORBAX Eclipse Plus C18, 2.1mm, 1.8μm, UHPLC guard column. The following LC-MS/MS conditions were obtained with mobile phase A (containing 5 mM ammonium formate, 0.1%formic acid in water) and mobile phase B (containing 5 mM ammonium formate, 0.1%formic acid in 75% methanol and 25% acetonitrile)

- Gradient settings:

Time [min]	Mobil phase A [%]	Mobile phase B [%]	Flow [mL/min]
0.00	88	12	0.2
5.00	88	12	0.2
5.01	50	50	0.2
16.00	0	100	0.2
17.00	0	100	0.2
17.01	88	12	0.2

- Injection volume: 6 μL
- Column temperature: 30°C

Ø MS/MS parameters:

Gas temperature [°C]	200
Gas flow [L/min]	8
Nebulizer [psi]	40
Sheath gas temp [°C]	350
Sheath gas flow [L/min]	11
Capillary [V]	3500
Nozzle voltage [V]	500

Ø Compound specific MS/MS parameters for mycotoxins, measured in ESI positive mode:

Analyte	Precursor Ion	Product Ion	Fragmentor [V]	Collision energy [V]	Polarity
Aflatoxin B1	313	285	165	24	+
		241		42	
Aflatoxin B2	315	287	165	28	+
		2,589		32	
Aflatoxin G1	329	243	160	28	+
		200		48	
Aflatoxin G2	331	313	165	24	+
		245		32	
Aflatoxin M1	3,291	2,731	163	24	+
		2,291		44	
		3,182		46	
HT-2	4,422	263	80	8	+
		2,151		8	
T-2	4,842	2,151	80	16	+
		1,851		24	
		1,891		20	
[$^{13}C_{17}$] Aflatoxin B1	3,302	3,011	156	24	+
[$^{13}C_{24}$] T-2	5,084	322	110	8	+

Analytical Methods

Feed samples were ground to pass through a 0.25 mm screen before analysis. Laboratory measurements were including Weender constituents (VDLUFA Section-III, 1993) and additionally starch, total sugars, calcium, phosphorus, and sodium. Analyses were in accordance with the methods issued by VDLUFA (Association of German Agricultural Inspection and research institutes) (dry matter: VDLUFA III 3.1; crude protein: VDLUFA III 4.1.1 modified according to macro-N determination (vario Max CN); crude fiber: VDLUFA III 6.1.4; crude ash: VDLUFA III 8.1; crude fat: VDLUFA III 5.1.1; starch: VDLUFA III 7.2.1; total sugars: VDLUFA III 7.1.1; calcium: VDLUFA VII 2.2.2.6). AFB1, the most common and biologically active aflatoxin, T2/HT2-toxin and ochratoxin in feeds were measured in an accredited external lab (D-PL-14016-01-00) according to FB-558-03-IAC-R7 9/17 (AFB1: LOD = 0.1 μg/kg), FB-535-04-R10 1/18 (T2/HT2: LOD = 1 μg/kg), and FB-554-78-R7 (Ochratoxin: LOD = 25 μg/kg). Blood cells were measured by flow cytometry. Blood concentrations in plasma were determined by using photometry. Sodium, potassium, and chlorides were measured by ionic liquid-polyacrylamide gel electrophoresis.

Statistical Evaluation

Results were presented according to the EFSA Guidance on Statistical reporting (EFSA Journal 2014; 12 (12):3908), descriptive statistics following Section 9.2.1 and results of statistical analyses in line with Section 9.2.2, respectively. Main analyses results were presented as point estimate and confidence interval. For all measurements taken at pen-level or at individual level, the basic statistical technique used was ANOVA with treatment as explanatory variable. After checking model assumptions, Tukey test was applied. Differences were considered significant when $P < 0.05$, whereas $P < 0.10$ were considered a near-significant trend. Analysis was performed with the software package SPSS (IBM SPSS Version 25).

Based on the supposed benefit of the test product on reducing the residue level of AFB1 in breast muscle the following power was estimated:

Study parameter	
Residue level of AFB1 in liver tissue	μg/kg dry matter
Mean group 1: AFB1	0.50
Mean group 2: AFB1 + MMDA	0.44
Sample size	5
Alpha	0.05
Post-hoc power calculation	83.5

Calculation based on the formula: n = f(α/2, β) × 2 × σ^2 / (μ1 − μ2)^2, where μ1 and μ2 are the mean outcome in the control and experimental group respectively, σ is the standard deviation, and f(α, β) = [Φ-1(α) + Φ-1(β)]^2, Φ-1 is the cumulative distribution function of a standardized normal deviate.

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Ethical Statement: The trial was performed in accordance with the Animal Welfare Act of Germany approved by the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales, LaGeSo, no. A 0439/17). Animals used in the study were raised and treated according to European Union Directive 2010/63/EU covering the protection of animals used for experimental or other purposes and according to the recommendation of Commission 2007/526/CE covering the accommodation and care of animals used for experimental and other scientific purposes. During the study, appropriate animal health and welfare inspections were carried out. The study animals were owned by Humboldt Universität zu Berlin.

Author contributions: This study was performed by Klaus Maennner and managed and designed by Jog Raj, Hunor Farkas and Marko Vasiljevic. Jog Raj, Marko Vasiljevic, Rakesh Kumar and Rajesh Asrani managed this study and wrote the scientific paper.

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DISCLOSURES

The authors declare no conflicts of interest.

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