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RESEARCH ARTICLE

Diagnosis of Subclinical Aflatoxicosis by Biochemical Changes in Dairy Cows under Field Conditions

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A B S T R A C T

Aflatoxins (AF) are potent mycotoxins with carcinogenic, teratogenic, and mutagenic potential. There is no agreement on the safe AF maximum residue levels established in different countries (5.0 to >20.0 µg/kg) to avoid feed toxicity in dairy cows and to protect the food chain. The objective was to establish a diagnosis of subclinical aflatoxicosis via changes in biochemical values during long-term exposure of AF low concentrations under field conditions. A cohort of 90 Holstein heifers were selected (395±10 kg/BW; 14-15 months) in a large dairy farm in the central Mexico highlands. Monthly samples of blood serum, feedstuffs, total mixed ration, and raw milk were obtained (26 months) and analyzed via spectrophotometric and HPLC methods. Dairy diets were naturally contaminated with AF (8.1±5.2 µg/kg). No cow showed clinical disease, but significant changes in biochemistry values were associated to AF intake at levels >5.0 µg/kg, especially a serum concentrations decrease in albumin, total protein and reduced glutathione; furthermore, an increase in prothrombin time, and in specific activity of AF metabolizing enzymes (glutathione S-transferase, γ-glutamyl transferase, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase). Raw milk samples were naturally contaminated with AF in milk (AFM_1; 43.1±24.0 ng/kg). A linear dose-response relationship between AF in feed and AFM_1 concentrations was observed (AFM_1=19.2+2.70(AF); P<0.01; R^2:62.1%). Moreover, reproductive failure and inter-pregnancy interval rates of cows exposed to higher AF concentrations (>10.0 µg/kg) were increased. These results suggested that in the long term, low amounts of AF exposure may lead to significant adverse effects consistent with subclinical aflatoxicosis.

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INTRODUCTION

Aflatoxins (AF) are secondary metabolites produced by the *Aspergillus* fungi, especially *A. flavus*, *A. nomius* and *A. parasiticus*; AF are potent mycotoxins, which contaminate worldwide a variety of agricultural commodities (FAO, 2004). The AF has widely demonstrated their carcinogenic, teratogenic and mutagenic potential; in addition, the AF develop immunodepression and synergistic effects with different pathogenic agents (Dubey *et al.*, 2007; Mozafari *et al.* 2017; Imran *et al*., 2019; Roshdy *et al*., 2020; Saleemi *et al*., 2020).

Ingested AF are quickly absorbed from the gastrointestinal tract and bio-transformed by the hepatic mixed-function oxidase system (cytochrome P_{450}), forming highly reactive epoxides. The epoxides bind the nucleophilic cell sites forming adducts; they have been found to be the cause of impaired protein formation, and alterations of blood coagulation as the prothrombin time (PT) (Benkerroum, 2020). The plasma enzyme specific activity (ESA) is increased when cell-bound enzymes are released into the bloodstream. Therefore, changes in ESA of γ-glutamyl transferase (GGT), ALT, AST and ALP, are suggestive of liver disorder induced by aflatoxin (Naseem

et al., 2018). The detoxification of AF epoxides occurs through its conjugation with a tripeptide called reduced glutathione (GSH), mediated by the ESA of glutathione-S-transferases (GST) (Lee *et al.*, 2010). AF-GSH conjugates are then excreted in bile as a less-toxic form (AF-N-acetilcysteine). Aflatoxin M_1 (AFM_1) is an AF hydroxylated derivative that can be excreted in cow's milk (Xiong *et al.*, 2015).

The clinical aflatoxicosis forms have been widely described as acute and chronic diseases (Benkerroum, 2020). Field outbreaks of acute aflatoxicosis occur in bovines exposed to high AF amounts (1.1-33.5 mg/kg) (Melo *et al.*, 1999, Pierezan *et al.*, 2012; Kaleibar and Helan, 2013; Umar *et al.*, 2015). Relevant clinical signs and lesions are related with fatty liver and coagulation impairment; chronic aflatoxicosis are similar but less evident; however, diagnosis is problematic because the clinical manifestations and lesions are unspecific and the delayed onset of them prevents their swift association with AF exposure (0.1-0.8 mg/kg) (Melo *et al.*, 1999; Umar *et al.*, 2015). In addition, it has been assumed that subclinical aflatoxicosis may be the result of ingestion of the lowest AF levels in contaminated feed over the long term. Although this toxicosis takes place most often, its existence is identified rarely because it occurs without obvious clinical signs (Pierezan *et al.*, 2012).

One of the most common strategies to control AF contamination is to set the maximum residue levels (MRL) or the action levels for AF, which are the maximum concentrations permitted of AF (<5.0 to >20 μg AF/kg) in food or feed (FAO, 2004). Although these dissimilar regulations are intended to protect human health and prevent toxicity in animals, there is no evidence of the effect that prolonged exposure to low levels of AF could have on animal health (Grenier and Applegate, 2013; Kemboi, 2016). Therefore, this form of continued exposure to AF below the MRL appears to be common under field conditions and may be causing subclinical forms of aflatoxicosis.

The objective was to establish a diagnosis of subclinical aflatoxicosis via changes in biochemical values during long-term exposure of AF low concentrations under field conditions.

MATERIALS AND METHODS

Survey design and herd management: A large dairy farm, officially certified as free of brucellosis and tuberculosis, was selected in central Mexico highlands. A cohort of 90 pregnant Holstein heifers was selected, and samples of feed, blood and raw milk were obtained at monthly intervals, during the first and second pregnancy (26 months). The animals were distributed by the farmer in separate open-air pens with free access to feeders, according to the milk production obtained level (high, medium, low, and dry).

The total mixed ration (TMR) was made with concentrate, corn silage, and hay, without added mycotoxin binders, mold inhibitors or antioxidants. The TMR was formulated to satisfy the nutritional requirements for milk production (Table 1) by ensuring adequate daily dry matter intake, metabolizable energy and crude protein.

Table 1: Feedstuffs and chemical composition of the total mixed ration of dairy cows

Item	Milk production level			
	High	Medium	Low	Dry
Milk production (kg/day)	30-40	20-29	10-19	--
Dry matter intake (kg/day)	25.0	18.6	16.2	14.4
Ingredient composition (kg/day DM)				
- Concentrate	10.5	7.6	5.3	3.3
- Corn silage	13.6	9.4	6.2	4.8
- Other forage (hay, silage or fresh)	0.27	1.10	4.0	5.8
- Mineral and vitamin mix[a]	0.51	0.49	0.54	0.46
- Sodium chloride	0.12	0.01	0.12	0.02
Chemical composition				
- Net energy for lactation (Mcal/kg)	1.55	1.47	1.37	0.97
- Crude protein (kg/d)	3.7	2.8	2.3	1.43
- ADF (%)	19.0	20.0	21.0	21.0
- NDF (%)	25.5	27.2	28.4	27.5

[a] The mineral and vitamin mix contained: 5.3-10.1 % Ca, 6.3-8.7 % Na, 4.6-8.3 % K, 0.22-0.46% S, 0.43-0.88 % P, 7893- IU of vitamin A/kg DM basis, and 1500- IU of vitamin E/kg DM basis. ADF = Acid Detergent Fiber; NDF = Neutral detergent fiber.

Feed, milk, and blood sampling: The concentrate, corn silage and TMR samples were obtained twice, directly from each batch. Feed samples were dried, homogenized, ground and were kept under refrigeration. Milk samples (300 mL) were proportionally obtained from every two-daily milking from each selected cow. Blood samples were collected by puncture of the medial coccygeal vein using vacutainer tubes without or with anticoagulant (sodium citrate). Samples were centrifuged to obtain serum or plasma and stored until analysis.

Aflatoxins analysis: The feed samples were processed in solid phase extraction tubes (Supelclean LC-CN, Supelco, USA), and eluates were derivatized with trifluoroacetic acid and injected into an HPLC system with fluorescence detector (Varian Associates Inc., Australia). The defatted milk samples were processed via immunoaffinity chromatography columns (AflaTest, Vicam, Milford, MA, USA) and the AFM_1 concentration was quantified by HPLC (Perkin-Elmer 200 series, USA). Quantitation of AF was performed using a calibration curve of purified AF (B_1, B_2, G_1, G_2 and M_1; Sigma Aldrich, USA).

The TMR was also analyzed for mycotoxins zearalenone (ZEN), ochratoxin (OTA), fumonisins (FBs) and deoxynivalenol (DON) via competitive ELISA kits (COKAQ 5100, 2000, 3000 and 4000; AgraQuant, Romer Labs, USA), according to the manufacturer's instructions.

Biochemical tests: Reduced glutathione was quantified by fluorometric method (Perkin-Elmer Luminescence Spectrometer LS-50B, USA). In a UV-Vis spectrophotometer (Varian DMS-80, Australia) the total protein (TP) and albumin (ALB) concentration in serum were determined, as well as ESA of GST, ALT, GGT, AST, ALA and ALP according to standard methods, and using appropriate diagnostic kits (Biosystems, Spain for Total Protein, Albumin/ALB, ALP-AMP, AST/GOT, ALT/GPT, γ-GT). Prothrombin time (PT) was performed using a commercial kit (Tcoag, TriniCLOT-PT Exel, Ireland).

Statistical analysis: The heifer's cohort sample size (n=86, plus 5.0%) was calculated for a finite population (N=839) without replacements to estimate the proportion of animals with a change in biochemical values (95% confidence interval, 10% accuracy). Each cow was

considered an experimental unit, from which repeated measurements were taken monthly.

Based on the quantification of the detected AF in TMR, the data of cows' biochemistry and AFM_1 were monthly assigned to group A, B, C or D (<5.0, 5.0-9.9, 10.0-19.9 and ≥20.0 µg/kg AF, respectively), according to usual MRL (FAO, 2004). Allowing these AF groups, one-way ANOVA and Tukey's honestly significant difference test procedure were used. The AF data were also analyzed by linear regression procedure to associate the variability of biochemistry values or AFM_1 as a single function of the AF concentration in TMR. The GLM procedure was used for evaluating the combined effect of AF in TMR, AF accumulated load, AF level group (group A, B, C or D), monthly change of AF level, and their respective interactions. The calculation of daily AFM_1 transfer to milk was performed as follows: *Transfer (%) = AFM_1 excretion (µg)/ AF consumption (µg) × 100.*

RESULTS

Mycotoxins in feed: During the study, almost all feed samples (308/312=99%) were naturally contaminated with detectable AF levels (Table 2, Fig. 1). The mean concentration of aflatoxins in TMR was 8.1±5.2 (range: ~0.0-84.2 µg/kg). In the majority of the TMR samples (82.7%; A-C groups), the AF concentrations detected were within the range allowed by local regulations (20 µg/kg) and by several international standards. Concentrations of OTA, FBs and DON (data not shown) were below the low limits of detection; while the ZEN concentration was detected sporadically only during months 12, 15, 23 and 26 of the study (56.3±38.2 µg/kg).

Biochemical changes: In this study, evidence of the association between long-term exposure to low AF concentrations and changes in biochemical parameters was found with a dose-response pattern (Fig. 2, panels a - i). Changes in biochemistry values were associated with the amount of AF intake at levels greater than 5.0 µg/kg, especially in the decrease of serum concentrations of ALB, TP and GSH; furthermore, an increase in the PT, and in specific activity of AF metabolizing enzymes (GST, GGT, ALT, AST, and ALP).

A gradual decrease in serum concentrations of ALB, TP, and GSH was observed, which were associated with an increase in the concentration of AF in the feed ingested by the cows (Fig. 2, panels a - c). A significant difference ($P<0.01$) in the serum concentration of ALB, TP, and GSH was also observed between the groups with the highest amount of AF (C and D) compared to the groups with the lower concentration of AF.

The ESA of GST, GGT, ALT, AST, ALP, and PT in serum showed an increase directly associated with increasing levels of AF in the diet ($P <0.05$); significant differences were also observed among groups B-D compared to group A, which had the least amount of AF in feed (Fig. 2, panels d-i). These differences were also noted when comparing the activity of C-D groups against reference values (Dubreuil and Lapierre, 1997).

The changes detected in the biochemical parameters were also simultaneously influenced by the combined effects of the concentration of AF in the diet, the accumulated load of AF, AF level group, and its change of AF level (Table 3). This combined model in GLM analysis was significant in all cases; however, the coefficient of determination R^2 was relatively small and less predictive of biochemical changes than the simple effects of the factor called AF group (Fig. 2).

AFM1 in milk: In this study, the concentration of AFM_1 in raw milk (43.1±24.0, ~0.0-80.0 ng/kg) showed a significant correlation with aflatoxins in TMR, as well as significant statistical differences among the groups A, B, C and D (Fig. 1). The transfer rate of AFM_1 to raw milk was relatively stable (0.71-0.78%) over the period under consideration.

Table 2: Aflatoxin frequency in batches of feedstuffs, total mixed ration (TMR), and raw milk of dairy cows naturally exposed to contaminated diet

Item	*Samples*[a] *(No)*	*Mean±SD* (µg/kg)	Aflatoxin frequency by level (% of samples) (<5)	(5-9.9)	(10-19.9)	(≥20)
Concentrate	28	7.6±7.5[b]	32.1	53.6	10.7	3.6
Corn silage	30	14.5±9.0[a]	6.7	23.3	46.7	23.3
Corn straw	10	3.8±1.6[b]	80.0	20.0	0.0	0.0
Triticale	12	6.9±4.9[b]	41.7	33.3	25.0	0.0
Other forages [b]	24	9.4±7.5[b]	25.0	41.7	25.0	8.3
TMR	208	8.1±5.2[b]	25.0	34.6	23.1	17.3
Raw milk	204	43.1±24.0	27.9	33.3	22.1	16.7

[a]Two samples were collected from each batch. [b]Alfalfa, oats or ryegrass as hay, silage or fresh. ([a-b]) Means of aflatoxin in feed with different letter are statistically different (Tukey HSD test, $P<0.01$).

Table 3: P-values and coefficient of determination of general lineal models of serum biochemical values and raw milk in dairy cows exposed to aflatoxins (AF) in total mixed ration (TMR) for 26 months

Serum biochemical values	Model	AF in TMR[a]	Load of AF[b]	AF group[c]	Monthly variation[d]	Coefficient of determination R^2 (%)
Albumin	<0.01	0.01	0.01	<0.01	<0.01	23.3
Total proteins	<0.01	>0.05	>0.01	<0.01	<0.01	23.2
Reduced glutathione	<0.01	0.03	<0.01	<0.01	<0.01	17.0
Glutathione S-transferase	<0.01	>0.05	<0.01	0.02	<0.01	22.8
γ-glutamyl transferase	<0.05	>0.05	<0.01	0.04	>0.05	1.7
Alanine aminotransferase	0.02	>0.05	>0.05	>0.05	0.01	1.9
Aspartate aminotransferase	<0.01	0.03	<0.01	<0.01	<0.01	10.7
Alkaline phosphatase	<0.01	<0.01	<0.01	<0.01	<0.01	28.8
Prothrombin time	<0.01	<0.01	>0.05	>0.05	<0.01	6.4
AFM_1 in raw milk	<0.01	<0.01	>0.05	<0.01	<0.01	83.1

[a]Aflatoxins quantified in total mixed ration (µ/kg). [b]Mean of monthly AF amount in TMR in 26 months (µg/kg). [c]AF in TMR, group A, B, C or D (<5.0, 5.0-9.9, 10.0-19.9 and ≥20.0 µg/kg), according to the usual maximum residue levels set in many countries. [d]Change (up or dawn) of AF group in comparison with previous month.

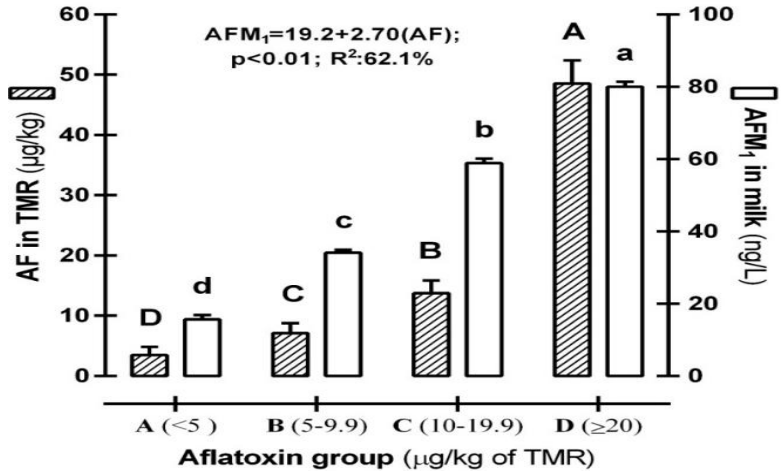

Fig. 1: Aflatoxins (AF) exposure in total mixed ration (TMR) of dairy cows and AFM_1 occurrence in raw milk (mean±SD). ($^{a-d}$) Means of AFM_1 in raw milk or AF in TMR ($^{A-D}$) with different letters are statistically different (Tukey HSD test, P<0.01).

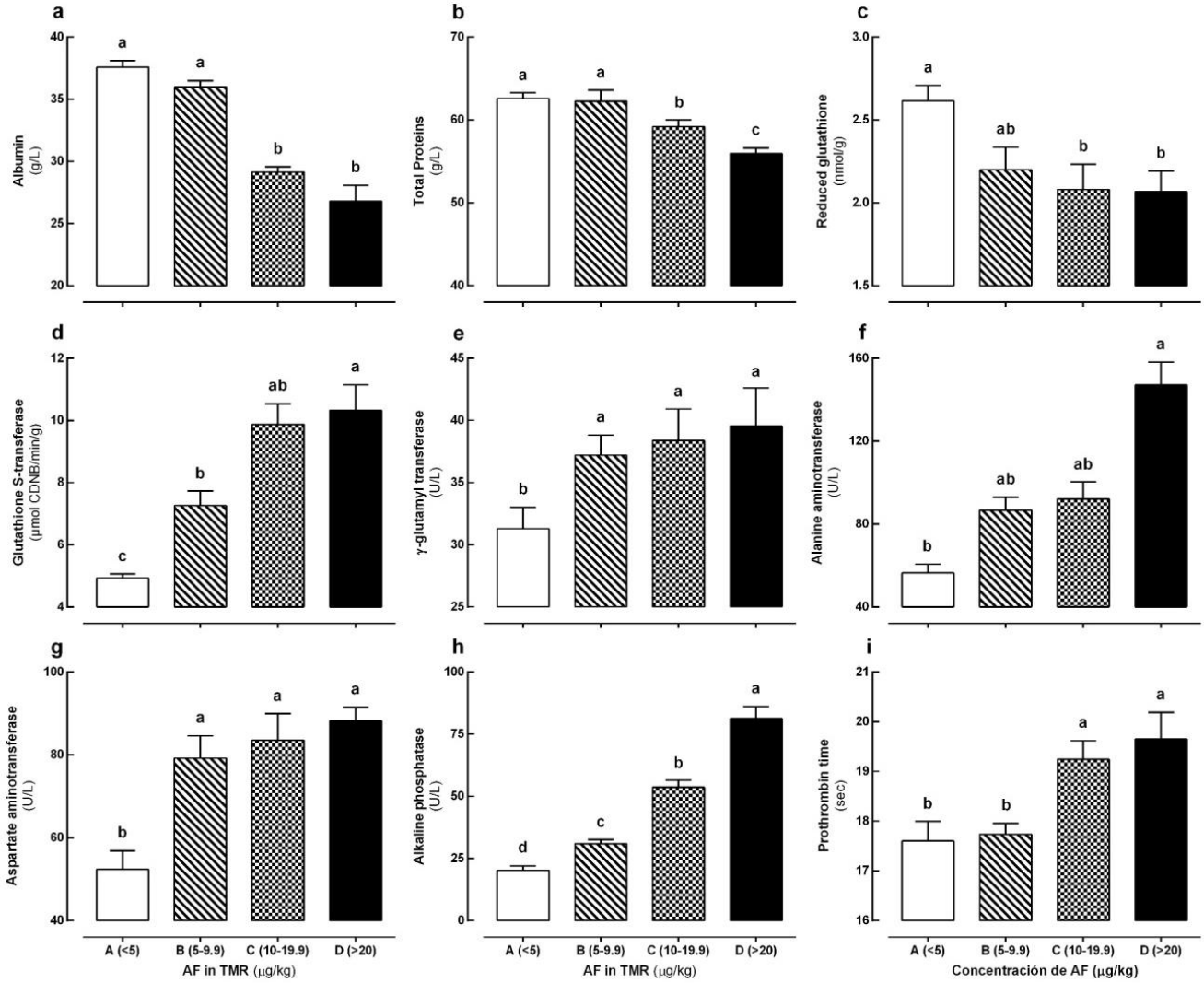

Fig. 2: Serum biochemical values (mean±SEM) in dairy cows naturally exposed to aflatoxin contaminated diet and linear regression as effect of aflatoxin (AF) in total mixed ration. CDNB = 1-chloro-2, 4-dinitrobenzene. ($^{a-d}$) Means with different letters are statistically different (Tukey HSD test, P<0.05).

Reproductive performance: Significant differences were detected in the reproductive performance of cows exposed to higher concentrations of aflatoxins in TMR (>10.0 µg/kg), in comparison with cows that ingested a lower amount; in particular, the rate of abortions was affected (17/29 = 58.6% vs 1/61 = 1.64%, respectively). The occurrence of abortion affected other reproductive parameters such as conception rate to first artificial insemination (16/29 = 55.2 vs 42/61 = 68.9 %) and culling rate due to reproductive failures (11/29 = 37.9 vs 1/61 = 1.64%). In addition, the inter-pregnancy interval was enlarged (396±7.2 vs 369±3.1 days). No alterations in the health of the cows with abortion were observed. The aborted fetuses had 120-240 days of gestation. The analysis of fetal tissue samples only detected *Neospora caninum* in six cases.

DISCUSSION

In this study, deleterious changes were observed in dairy cows naturally exposed, by long term, to low levels of aflatoxins in their diet. These changes were consistent with the occurrence of subclinical aflatoxicosis because no cow showed clinical disease, but blood biochemistry had evident alterations besides the presence of AF-M_1 in milk and poor reproductive performance. To our knowledge, the long term (>2 yr.) of natural exposure to low AF concentrations represents the first report of this approach in dairy cows. The timely diagnosis of the effects caused by the presence of AF in feed is highly relevant in the dairy industry to decrease the large and negative impact that this mycotoxin produces on performance, animal health and on milk contamination with AFM_1.

In our study, virtually all TMR samples had some concentration of AF. The amount of AF in the dairy cows' diet were comparable to those obtained in other field studies in feed of dairy cows in the Central Mexican Highlands and worldwide (Walte *et al*, 2016; Rangel *et al.,* 2020). Due to the AF persistent contamination, the mycotoxin binders have been widely used up to tolerable levels (Min *et al.,* 2020), complementing other contamination surveillance, prevention, and remediation strategies (Walte *et al*., 2016; Haque *et al*., 2020). Therefore, the results of this study agree with the fact that low AF concentration in feed consumed by dairy cows is frequent.

The detected differences in this study were attributed to the natural variation of AF contamination in each batch of feed used to prepare the cow's diets. Furthermore, the time and amount of AF in which each of the cows was exposed to AF was also under natural variation. The changes detected in the biochemical parameters were also simultaneously influenced by the combined effects of the concentration of AF in the diet, the accumulated load of AF, and its change of AF level (Table 3).

Similarly, in other studies (Reyes-Velázquez *et al.*, 2009; Rangel *et al.*, 2020) on the natural contamination by OTA, FB, DON and ZEN in dairy feed, low concentrations were found. Exposure to ZEN high concentration (>400 µg/kg) in feed can result in decreased cattle reproductive performance (Kemboi, 2016); however, the observed ZEN concentration was eight times lower at that concentration, suggesting that the reproductive changes detected in this study were not caused by ZEN.

A gradual decrease in serum concentrations of ALB, TP and GSH was observed, which were associated with an increase in the concentration of AF in the feed. Pierezan *et al.* (2012) and Bhatti *et al.* (2016) also reported that AF decreases the concentration of ALB and TP in animals with AF toxicity. Hence, the decrease in serum ALB and PT concentration detected in our study suggests that the chronic consumption of small concentrations of AF can induce a reduction in protein serum concentration. On other side, the GSH conjugation is the main mechanism to prevent the binding of AF-8,9-epoxide to nucleic acids and proteins of subcellular organelles (Benkerroum, 2020). Therefore, the results of our study suggest that decreased GSH levels were observed as a response to long-term exposure of AF low concentrations.

The ESA of GST, GGT, ALT, AST and ALP showed a significant increase associated with the increase in the amount of AF in TMR. These enzymes have been shown to be involved in the AF detoxification process and may be related as a general indicator of liver disease in cattle (Liu *et al*., 2012; Naseer *et al.,* 2016; Naseem *et al.,* 2018). In our study, the increase in ESA of GST, GGT, ALT, AST and ALP in dairy cows also suggests an adverse effect of long-term exposure to low concentrations of AF.

In this study, an increase in PT was also detected, which was significantly associated ($P<0.01$) with the concentration of AF in the TMR. The increase of PT has been widely reported in cattle blood coagulation impairment by dicumarol (McGuffey, 2017). The coumarin nucleus, together with a reactive bifuran system, explains the potential of aflatoxins to alter blood clotting and cause hemorrhagic lesions in cattle poisoned with large amounts of AF. Therefore, the increase in the TP of our study suggests that the consumption of AF in the feed increases the hazard of suffering alterations in the blood coagulation.

The concentration of AFM_1 in raw milk showed a significant correlation with aflatoxins in TMR, and the transfer rate of AFM_1 to raw milk was relatively stable (0.71- 0.78%). Comparable transfer rate of AFM_1 have been demonstrated in Holstein cows experimentally exposed to AF-contaminated feed (Masoero *et al.,* 2007; Xiong *et al.,* 2015).

In this study, significant differences were detected in the reproductive performance of cows exposed to higher concentrations of aflatoxins in TMR (>10.0 µg/kg), in comparison with cows that ingested a lower amount. It has previously been suggested that constant exposure of dairy cows to AF can trigger pathological changes in immunity, endocrine system, reactivation of pathogens and reproductive failure (Dubey *et al.,* 2007; Mozafari *et al.,* 2017). The alterations detected in our study differ from acute aflatoxicosis, which occurs in dairy cows exposed to AF large amounts and there are associated with systemic and digestive clinical findings (weight loss, depression, ataxia, recumbency, photosensitization, jaundice, anorexia, diarrhea, dysentery, rectal prolapse, etc.), mortality with hemorrhagic lesions in intestine, liver and kidney (Kaleibar and Helan, 2013; Elgioushy *et al*., 2020; Kemboi *et al.,* 2020). Our results also differ from chronic aflatoxicosis in cattle AF exposed to moderate amounts of for prolonged periods, which is characterized by altered ruminal, hepatic, reproductive and immune functions (decreased milk production, poor feed conversion, immunosuppression, reproductive failure, lameness, etc.) (Ogunade, *et al.,* 2018; Kemboi *et al*., 2020). In this study are reported in summary, the deleterious effects on serum biochemistry, AFM_1 in milk and alterations in reproductive parameters suggested that apparently healthy dairy cows had subclinical aflatoxicosis induced by long-term ingestion of AF, despite AF concentrations they were below national or regional action levels.

Conclusions: This study describes changes in biochemical parameters of apparently healthy dairy cows related to long-term ingestion (26 mo.) of low amounts of AF in naturally contaminated feed. In dairy cows that ingested AF levels >5.0 µg/kg of feed, alterations in the coagulation process, hypoproteinemia and increased serum activity of AF detoxification enzymes were detected. Moreover, transfer and excretion of AFM_1 in milk in a dose-response pattern with the AF concentration in feed was observed. These results suggested that long-term consumption of feed containing low concentrations of AF could result in adverse effects on animal health and performance, which was consistent with subclinical aflatoxicosis.

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Authors contributions: EHV, AGV, CCV, TQT and ROM conceived and designed the study. EHV, AGV, EJRM, LEME and FJJ executed the experiment and analyzed the feed, milk, and serum samples. MCMS, LEME and FJJ analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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