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Effect of chemical- and toxin binder-treated cotton seed cake on milk production, milk composition, and aflatoxin concentration in milk of Nili Ravi lactating buffaloes

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Abstract: Twelve multiparous Nili Ravi buffaloes were assigned to 1 of 3 treatments in a randomized, replicated 3 × 3 Latin square design to evaluate the effect of chemical (calcium propionate)- and toxin binder (sodium bentonite)-treated cotton seed cake (CSC) on milk yield and transfer of milk toxin (M_1). Periods (20 days) were allocated for each treatment. In the 1st treatment (control), CSC with 100 µg/kg of aflatoxin B_1 (AFB_1) was fed to buffaloes. The 2nd and 3rd treatments were 0.5% calcium-propionate–treated CSC and 1.0% sodium-bentonite–treated CSC, respectively. Each group had 4 multiparous buffaloes. The buffaloes given 0.5% calcium propionate CSC had less M_1 (0.29 µg/kg) in milk compared to the 1.0% sodium bentonite (0.30 µg/kg) treatment group. All treatments had an insignificant effect ($P > 0.05$) on CSC intake (4.62 kg), milk production (7.0 L per day), and milk composition. To conclude, oral intake of calcium propionate reduced the transfer of aflatoxin from rumen to milk.

Key words: Aflatoxin, buffalo, chemical detoxificant, commercial, cotton seed cake, milk toxin

1. Introduction

Aflatoxins (AFs) are secondary metabolites which are formed by *Aspergillus* species [1]. AFs are of mainly two types: aflatoxin B_1 (AFB_1) and aflatoxins B_2 (AFB_2). These metabolites are immunosuppressive and carcinogenic in humans as well as animals [2]. Feed is usually contaminated with AFB_1 and AFB_2 pre- and postharvest. Cotton is a leading earning crop in South Asia. In Pakistan, total cotton production was 9,917,000 bales in 2015–2016 [3]. Cotton seed cake (CSC) is a by-product of the cotton seed oil industry. It is a readily available protein ingredient for livestock feed in Pakistan due to its moderate protein and high oil contents [4]. Contamination of CSC with AFB_1 and AFB_2 is a major issue in the feed industry of Pakistan. Cotton seed cake has a high incidence of AF [5]. Postharvest contamination due to inadequate processing and storage is the larger problem in the case of CSC [6]. When AF-contaminated diets are given to lactating buffaloes and cattle, the toxin is metabolized in the liver into a hydroxylated derivative called AFM_1, transported to the mammary glands via blood, and shifted to milk [7].

All of this points to the need for practical and cost-effective measures to reduce mycotoxin problems. A number of approaches have already been used to counteract this menace, though only a few have real practical applications [8]. Physical treatment, including nonchemical washing, polishing, mechanical separation, flotation, and autoclaving are used as a first option [9]. Other methods include detoxification, such as sterilization, pasteurization, and other heat treatments [10]. Solvent extraction is another method used in oiled seed products. The effect of heat treatment on detoxification of aflatoxin is 50%–80% [11]; however, it depends on the raw material moisture contents. Chemical treatment includes the use of calcium hydroxide, ammoniation, copper sulfate, sodium bisulfide, and various acids to decrease the aflatoxin [12]. Chemical compounds like acids, bases, oxidizing agents, gases like ammonia, and aldehydes can be used for structural degradation when adopting chemical methods [13]. It was also reported that chemical detoxification can reduce the level of aflatoxin up to 93%–95% [14]. Treating milk with hydrogen peroxide instead of pasteurization reduces the AFM_1 in cheese made from the milk [15]. Among chemical detoxificants, calcium propionate has shown good results for decreasing aflatoxin [12].

Previous studies are not clear about the exact concentration of various chemicals used to reduce aflatoxin in cattle feed. However, some levels have been selected

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based on earlier work to assess their efficacy at detoxifying aflatoxin. The third option is mycotoxin binders which are extensively used by the animal feed industry [8].

It is crucial to research and evaluate strategies to counter the problem described above. Chemical detoxificants such as calcium propionate are one option. In order to mitigate this problem, the current study compares calcium propionate with a commercial toxin binder (sodium bentonite). An effective feed additive must reduce the bioavailability of AF without affecting the performance of the animal or the nutritional content of the animal products.

The aim of this research was to reduce AFM_1 concentration in milk and to determine responses in milk composition by introducing calcium propionate and sodium bentonite into AF-contaminated CSC.

2. Materials and methods

2.1. Experimental animals

This research was carried out in January 2019 at Buffalo Research Institute Pattoki, Pakistan (31.02 °N, 73.85 °E, and 186 m altitude). Twelve Nili Ravi buffaloes of second and third lactation with an average weight of 600 ± 50 kg were used in this study. The buffaloes were individually fed and drinking water was available around the clock.

2.2. Experimental design, treatments, and feeding

Buffaloes were divided into three groups (four buffaloes in each group) in 3 × 3 Latin squares. The test products were calcium propionate (CaP 0.5%) and sodium bentonite (NaB 1%) (Riedel-de Haen Company, Germany). Cotton seed cake, corn silage, and wheat straw containing 100 µg/kg of AFB_1 was used as the control treatment, and in the subsequent treatments, 1 level each of calcium propionate (i.e. 0.5%) and 1% sodium bentonite were used. The buffaloes were fed individually, and the diets were offered in a restricted amount. The total duration of experiment was 60 days, and each period was 20 days with the first 7 days used for dietary adjustment. The AF content was initially determined by ELISA analysis. Every effort was made to ensure that treatment was equally distributed into every part of the CSC.

2.3. Micro aspects of standardization and other practices

One aspect of the first part of this experiment was the estimation of aflatoxin. This was done using ELISA kit method before and after the treatment. Every effort was made to ensure that treatment was equally distributed to every part of the CSC. The blue-bordered dilution strips required for samples and the standards were placed in a microwell strip holder. An equal number of antibody-coated strips were also placed in a microwell strip holder. A multichannel pipette was used to deliver 100 mL of the conjugate into each of the blue-bordered dilution wells. Then 50 mL of the sample and the standard were placed in the dilution well containing the HRD conjugate base. The multichannel was used to mix the sample by carefully pipetting it up and down three times. Then, 100 mL of the mixture was immediately transferred to the antibody-coated plate and incubated for 15 min at room temperature. The antibody-coated plates were decanted and washed five times with deionized water. A 100 mL portion of the substrate was placed on the antibody-coated plate and incubated. A stop solution (the solution which leads to color change from blue to yellow) of 100 mL was introduced into each antibody-coated plate. The microwell strips were subsequently analyzed using a microwell reader. The optical density reading for each microwell was recorded. Standard curve generation used aflatoxin concentrations in the range of 0–6 ng/g.

Diets were prepared using CPM-Dairy 3.0.10 (Cornell University, Ithaca, NY, USA; University of Pennsylvania, Philadelphia, PA, USA), on the basis of the Cornell Net Carbohydrate and Protein System (CNCPS) 5.0 [16]. The composition and nutritive values of the diets are presented in Table 1. Briefly, the diets consisted of corn silage, CSC, wheat straw, and mineral premix. Feeding was done once in the morning at 9:00 am, while milking was done two times a day at 5:00 am and 3:00 pm.

2.4. Sample collection, preparation, and analysis

Feed sampling, samples of CSC, wheat straw, and fodder were collected on day 18 of each period and subsequently pooled by period. Samples of feed were subjected to drying at 65 °C in a hot air oven to determine dry matter (DM) at the Nutrition Laboratory of Buffalo Research Institute, Pattoki District Kasur, Pakistan. In the last 3 days of each period, apparent DM, crude protein (CP), and neutral detergent fiber (NDF) digestibility tests were conducted. The digestibility trial was conducted using the total collection method [17]. The feces of each animal were collected daily, weighed, and mixed thoroughly, and 20% of the total was sampled and dried at 55 °C. At the end of each collection period, dried fecal samples were composited, and 10% of the composited samples were taken for analysis. The analysis includes DM [18] and NDF [19]. Individual milk yield was measured and recorded daily, and measurements from the first 15 days of each period were used to evaluate milk production. Milk samples were collected on days 14 and 15 of each treatment to investigate milk composition. Analysis of milk samples for fat, lactose, solid not fat (SNF), milk density, and protein were completed in the nutrition laboratory, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki using the ultrasonic milk analyzer Milkotester Master (Milkotester LTD, Belovo, Bulgaria).

2.5. Aflatoxin (AFM_1) detection in milk

Milk samples in sterilized plastic bottles were sent in an ice-packed cooler to the WTO laboratory, University of Veterinary and Animal Sciences, Lahore where they were stored at –20 °C until further analysis for AFM_1.

Table 1. Ingredient, chemical composition, and predicted nutritive value of feed.

Ingredient	% DM	AF
Corn silage	54.47	75.95
Wheat straw	23.86	12.66
Cotton seed cake	19.09	10.13
Mineral premix	2.58	1.26
Total	**100**	
Nutrient, analyzed content		
Dry matter	100	48.8
Crude protein (%)	9.55	4.66
ADF (%)	31.97	15.6
NDF (%)	46.87	22.87
EE (%)	4.09	1.99
Ash (%)	7.92	3.86
NFC (%)	33.3	16.25
Predicted using CNCPS system		
RUP (%CP)	37.53	37.53
RDP (%CP)	62.47	62.47
RDP (%)	5.96	2.91
ME (MCal/kg)	2.12	1.03
NEl (MCal/kg)	1.36	0.67
Sugar (%)	4.05	1.98
Starch (%)	20.47	9.99

AF: As fed basis
ADF: Acid detergent fiber
NDF: Neutral detergent fiber
EE: Ether extract
NFC: Nitrogen-free extract
RUP: Ruminally undegradable protein.
RDP: Ruminally degradable protein.
NE_L: Net energy for lactation.

2.5.1. Chemicals and reagents

Acetonitrile (HPLC grade) from Sigma–Aldrich (Steinheim, Germany) was used for AFM_1 analysis. The immunoaffinity columns $AflaM_1$ TM HPLC were taken from VICAM (Watertown, MA, USA). The water used for analysis was double distilled with a Millipore water purification system (Bedford, MA, USA). The AFM_1 standard (10 µg/mL in acetonitrile) was purchased from Supelco (Bellifonte, PA, USA). All other chemicals used were of analytical grade.

2.5.2. Extraction procedure

The AFM_1 in milk was extracted using the method described by Dragacci et al. [20].

2.5.3. Determination of AFM_1 with fluorescence detection

Each sample was processed for determination of AFM_1 with the HPLC system of Agilent 1100 series (Agilent, USA) using the official AOAC method 2000.08 [19]. The HPLC system was equipped with a LAS G1313A autosampler and a FLD G1321A fluorescence detector with excitation and emission wavelengths of 365 nm and 435 nm, respectively. The retention time for AFM_1 was 6.1 min.

2.6. Statistical analysis

Data were analyzed using the mixed procedure of SAS University Edition (SAS Institute Inc., Cary, NC, USA) using the main effects of period and treatments; buffaloes were designated as a random effect in the model. The following mathematical model was used for the analysis:

$$Yijk = \mu + Buffi + Perj + Treatk + \varepsilon ijk$$

The data were analyzed using GLM procedures [21]. Treatments, periods, and buffaloes were the main effects.

3. Results and discussion

3.1. Dry matter intake

The dry matter intake (DMI) is given in Table 2. Dry matter intake of different treatments was the same ($P > 0.05$). Dry matter intake of control, CaP 0.50%, and NaB 1.0% were 15.67 kg, 15.67 kg, and 15.66 kg, respectively. Neither treatment had a significant effect on DMI. Supplementation with chemical detoxificant and commercial toxin binder did not increase or decrease the DMI. Observations of this study on DMI are in line with previous studies [1,22,23], which pointed out that providing an AFB_1 diet with or without treatment had no effect on DMI. This was also the case in our experiment. Maki et al. [1] used calcium montmorillonite clay to counter AFM_1 in the milk of Holstein cows and found no decline in DMI. Our study is also in agreement with Sulzberger et al. [24] in which different levels of clay (0.5%, 1%, and 2%) were used in an AF challenge diet. None of the concentrations had a significant ($P > 0.05$) effect on DMI. Our study was conducted in buffaloes with calcium propionate and sodium bentonite; however, no difference was observed.

3.2. Cotton seed cake intake

The cotton seed cake intake was nonsignificant ($P > 0.05$) among the treatments. Cotton seed cake intake was lower (4.57 kg) in control treatment compared to other treatments. The CSC intake improved with the addition of calcium propionate (CaP 0.5%) to 4.65 kg and 4.64 kg for NaB 1.0%. Cotton seed cake intake posed a nonsignificant difference ($P > 0.05$) among treatments. This is in accordance with the findings of Jouany [9] who reported that an AFB_1-contaminated diet (0.02 mg/kg) reduced feed intake in Friesian cattle. Our results are in line with the conclusions of Pasha [25] which confirmed a decline in

Table 2. Effect of dietary addition of calcium propionate and sodium bentonite on the dry matter intake, milk yield, milk composition ,and milk toxin (AFM_1) of Nili Ravi lactating buffaloes consuming an aflatoxin (AF) challenge diet.

Item	Treatments*			SEM	P-value
	0	CaP	NaB		Treat
DMI, kg/d	15.67	15.67	15.66	0.080	0.50
CSC intake, kg per day	4.57	4.65	4.64	0.100	0.454
Milk yield, L per day	7.72	7.46	7.25	0.384	0.15
Fat content, %	7.98	7.64	7.62	0.230	0.49
Milk protein%	3.35	3.40	3.41	0.072	0.69
SNF%	8.02	8.17	7.84	0.075	0.69
Milk density%	23.25	23.05	25.06	0.575	0.97
Lactose%	4.38	4.37	4.52	0.069	0.23
AFM_1 µg/kg	1.70[a]	0.29[b]	0.30[b]	0.006	0.0001

SEM; standard error of mean.
DMI: dry matter intake, CSC: cotton seed cake, SNF: solid not fat, AFM_1: aflatoxin in milk
*0: calcium propionate and sodium bentonite 0%; CaP, calcium propionate 0.50%; NaB, sodium bentonite 1%.
Different letters in the same line indicate significant differences.

feed intake in Sahiwal dairy cows given 500 µg/kg of AFB_1-infected feed without any treatment or supplementation.

3.3. Milk yield

Milk yield was similar (P = 0.15) across the treatments with an average of 07.47 L per day. The differences in this study were minor. Milk yield was 7.72 L per day for treatment without supplementation and 7.46 L per day and 7.25 L per day for supplementation with CaP 0.5% and NaB 1.0%, respectively. Milk yield showed a downward trend with supplementation, but this trend was statistically nonsignificant (P > 0.05). Milk yield was similar (P = 0.15) across treatments with an average of 7.47 L per day. Kutz et al. [23] used sorbent and reported deleterious effects on milk yield. Sulzberger et al. [24] used clay for aflatoxin (M_1) in milk. Milk yield was P = 0.154 with the AFB_1 challenge in feed and treatment with clay. In this study we used calcium propionate and sodium bentonite as detoxificants, and the differences were minor.

3.4. Milk composition

Our study demonstrated similar values in fat (P = 0.49), protein (P = 0.69), SNF (P = 0.69), milk density (P = 0.97), and lactose (P = 0.23) across all treatments (Table 2). No remarkable change was noticed in all treatments irrespective of the addition of detoxificants. Milk composition in terms of milk fat, protein, SNF, density, and lactose were the same across all treatments. Our study is in full agreement with other experiments in which neither an AF-contaminated diet nor supplementation had any effect on the milk composition of dairy cows [22,23]. According to these studies, none of the treatments changed milk composition in Holstein cows. This experiment is also in full agreement with Smith et al. [26], a study that was conducted in goats; no change in milk composition was noted in dairy goats given feed containing calcium aluminosilicate. Our results for milk composition of buffaloes given chemical detoxificant (calcium propionate) and commercial toxin binder (sodium bentonite) were similar to studies conducted in ewes in which yeast was used to reduce AFB_1 in feed and AFM_1 in milk. No change in milk composition was reported [27,28].

3.4.1. Aflatoxin in milk (AFM_1)

AFM_1 content in milk is presented in Table 2 and Figure. The AFM_1 across all groups was different (P < 0.05). The AFM_1 was significantly less in CSC treated with CaP 0.5% (0.29 µg/kg), followed by NaB 1.0% (0.30 µg/kg). Specifically, the transfer rate was reduced from 1.70% (control) to 0.29% (CaP 0.5%). The AFM_1 among all groups was different (P < 0.05). The AFM_1 was significantly lower in CSC treated with CaP 0.5% (0.29 µg/kg), followed by NaB 1.0% (0.30 µg/kg). Specifically, the transfer rate was reduced from 1.70% (control) to 0.29% (CaP 0.5%). Similar transfer rates have been reported for dairy cows consuming AF-contaminated diets [29,30], and these reports noted that milk toxin and its transfer rate were

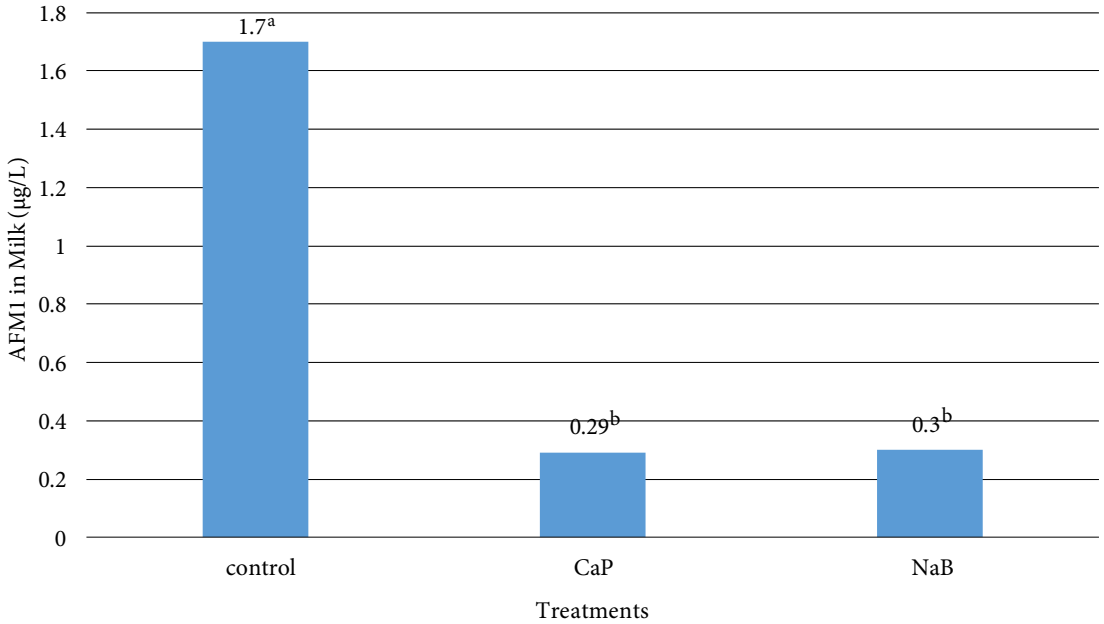

Figure. Graph representing the interquartile range and distribution of the data from the 12 buffaloes sampled at independent time frames in the Latin square design. Control = Cotton seed cake without any treatment. CaP = Cotton seed cake treated with 0.5% calcium propionate. NaB = Cotton seed cake treated with sodium bentonite.

Table 3. Effect of dietary addition of calcium propionate and sodium bentonite on the digestibility of nutrients of Nili Ravi lactating buffaloes consuming an aflatoxin (AF) challenge diet.

Item	Treatments*			SEM	P-value
	0	CaP	NaB		Treat
DM digestibility%	62.50	62.50	62.25	0.020	0.96
CP digestibility%	63.50[a]	67.75[b]	67.16[b]	0.201	0.001
NDF digestibility%	35.41	35.66	36.58	0.420	0.31

SEM; standard error of mean.
DM: dry matter, CP: crude protein, NDF: neutral detergent fiber.
*0: calcium propionate and sodium bentonite 0%; CaP, calcium propionate 0.50%; NaB, Sodium bentonite 1%.
Different letters in the same line indicate significant differences.

significantly reduced by using the commercial toxin binder calcium aluminosilicate in animal diets. In our study different toxin binders were used; however, their mode of action was the same. Calcium aluminosilicate and sodium bentonite have adsorptive characteristics and showed the same results. The normal mode of action for these adsorbents is to sequester AF in the rumen; this tightens the bonding with the molecules of AF and does not spare AF [23]. Nili Ravi buffalo, a native breed, was used in this experiment. No difference in breed was observed, as commercial toxin binders have the same mode of action in cattle and buffaloes in term of reduction of AFM_1 in milk.

3.4.2. Digestibility of nutrients

The nutrient digestibility (Table 3) was the same ($P > 0.05$) for dry matter ($P = 0.96$) and neutral detergent fiber ($P = 0.31$), and it was different ($P < 0.05$) for protein ($P = 0.001$). The AF challenge and detoxificants only affected the digestibility of protein. Our study is in line with Kiyothong et al. [31] who reported improved digestibility of protein while supplementing with mycotoxin deactivator. The reason behind this is that these detoxificants enhance the digestibility of the main nutrients, i.e. protein. Our results are in line with Stanford et al. [32], who also mentioned improved digestibility of protein in lambs fed

with a Biomin-treated diet. The reason for this is that AF hinders the digestibility of nutrients. When an adsorbent is added to feed with AF content, the adsorbents attach to the AF molecules. As a result, digestibility of nutrients is improved. Both sheep and buffaloes are ruminants, and their digestive physiology is not markedly different.

4. Conclusion

Cotton seed cake is a readily available protein source in Pakistan, but the incidence of AF in this ingredient limits its usage in livestock feed. Different strategies are being explored to mitigate this problem. This study compared calcium propionate (0.5%) with a commercial toxin binder (sodium bentonite 1%) to find the best possible solution to this menace. In our study both calcium propionate and sodium bentonite were quite effective at reducing AFM_1 in milk. Neither had harmful effects on the health of the buffaloes or their milk production and composition. More studies are required to explore the use of chemical detoxificants and commercial toxin binders in other feed ingredients which have high levels of aflatoxin, such as 30% maize gluten meal. The interaction of blood metabolites with AFM_1 and the effects in buffaloes and other species are also important topics for future studies.

Statement of animal rights

The entire study was carried out according to the regulations of the ethical committee for animal welfare of the University of Veterinary and Animal Sciences Lahore, Pakistan.

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