Effects of 3 sequestering agents on milk aflatoxin M₁ concentration and the performance and immune status of dairy cows fed diets artificially contaminated with aflatoxin B₁

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ABSTRACT

This study examined whether adding 3 mycotoxin-sequestering agents to diets contaminated with aflatoxin B₁ (AFB₁) would reduce milk aflatoxin M₁ (AFM₁) concentration, and improve the performance and alter immune status of dairy cows. Fifteen lactating dairy cows were used in an experiment with an incomplete crossover design including four 28-d periods. Treatments included a control diet (C), a toxin diet (T; 1,725 µg of AFB₁/head per day; 75 µg/kg), and diets containing the toxin and 20 g/head per day of a proprietary mixture of *Saccharomyces cerevisiae* fermentation product containing a low (SEQ1) or high (SEQ2) dose of a chlorophyll-based additive, or a low dose of the chlorophyll-based additive and sodium bentonite clay (SEQ3). Sequestering agents were top-dressed on the total mixed ration (TMR) daily in each period, and AFB₁ was dosed orally in gelatin capsules before the TMR was fed on d 21 to 25. Milk was sampled twice daily on d 20 to 28 and plasma was sampled on d 20 and 25. Sequestering agents did not affect milk AFM₁ concentration during the toxin-dosing period. However, after AFB₁ was withdrawn, the sequestering agents reduced the time required (24 vs. 48 h) to reduce the milk AFM₁ concentration below the Food and Drug Administration action level of 0.5 µg/kg. Feeding T instead of C tended to reduce milk and fat-corrected milk yields, but feeding SEQ1 prevented these effects. Red blood cell count and hemoglobin concentration were reduced by feeding T instead of C, but not by feeding SEQ1, SEQ2, or SEQ3. The mean fluorescence intensity of antibody staining for 2 leukocyte adhesion molecules, L-selectin (CD62L) and β-integrin (CD18), tended to be greatest when SEQ1 and SEQ3 were fed. Plasma acid-soluble protein concentration was decreased by feeding SEQ1, SEQ2, and SEQ3 instead of T. Sequestering agents had no effect on milk AFM₁ concentra-

tion, but they reduced the time required to reduce milk AFM₁ concentration to a safe level after withdrawal of AFB₁ from the diet. Only SEQ1 prevented the adverse effects of AFB₁ on milk and fat-corrected milk yields.

Key words: aflatoxin, milk, sequestering agent

INTRODUCTION

Mycotoxins are a group of toxic compounds produced by fungi on growing forages or stored feeds that can reduce the feed intake, growth, health, and reproductive performance of livestock and humans. Aflatoxin B₁ (AFB₁) has been the most widely studied and problematic mycotoxin in dairy cows because its metabolite, aflatoxin M₁ (AFM₁), can be transferred to milk (Diaz et al., 2004). Up to 166,000 cases of hepatocellular carcinoma per year in humans have been attributed to aflatoxins (Liu and Wu, 2010). Due to the high consumption of milk by humans, particularly infants, the Food and Drug Administration (FDA) regulates the maximum permissible amount of AFM₁ in milk at 0.5 µg/kg and AFB₁ in dairy feeds at 20 µg/kg (FDA, 2000). The maximum milk AFB₁ concentration allowed by the European Commission is 0.05 µg/kg (EFSA, 2004).

Various approaches have been employed to reduce the risk of aflatoxin ingestion by livestock and the resultant aflatoxicoses. Ozonization and ammoniation can minimize the production and effects of the toxins in corn and cottonseed meal (CAST, 2003), but these approaches are costly and time-consuming (Kutz et al., 2009), so they are not typically used on dairy farms. One of the more recent approaches is to use adsorbents that bind aflatoxin in the gastrointestinal tract and reduce its bioavailability (Huwig et al., 2001, Phillips et al., 2002). Clay-based aflatoxin-sequestering agents have shown the potential to bind aflatoxins and impair their gastrointestinal absorption (Kabak et al., 2006), but high doses may reduce utilization of vitamins and minerals (Chestnut et al., 1992). The outcomes of studies testing the effectiveness of adsorbents at reducing the transfer of the toxins to milk have been inconsistent

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(Kabak et al., 2006). Diaz et al. (2004) reported that 3 sodium bentonite products reduced the concentration of AFM₁ residues in milk by 50 to 65%. Another study (Stroud, 2006) compared 8 binding agents, including 3 combinations of yeast and clay products and 5 clay products, and revealed that 1 of the former and 3 of the latter reduced the transfer, secretion, or concentration of AFM₁ in milk. A recent study by Xiong et al. (2015) showed that a product based on yeast and sodium montmorillonite reduced the transfer of aflatoxin to milk at a low dietary dose (20 µg/kg) but had no effect at a high dose (40 µg/kg). We have shown that a high dose (1% of diet DM), but not a low dose (0.2% of diet DM), of montmorillonite clay reduced the transmission of dietary AFM₁ to the milk of dairy cows (Queiroz et al., 2012). There is clearly a need to continue to evaluate the effects of aflatoxin-sequestering agents on milk AFM₁ concentration, as well as on milk yield and quality. The objective of this study was to examine whether dietary addition of 3 *Saccharomyces cerevisiae* fermentation product-based mycotoxin-sequestering agents would reduce milk AFM₁ concentration and improve the performance and health of dairy cattle that were fed diets artificially contaminated with AFB₁.

MATERIALS AND METHODS

Cows, Treatments, and Design

Cows in this study were cared for according to the University of Florida Animal Research Committee protocols, which were developed from guidelines outlined in the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). Fifteen lactating multiparous Holstein cows in mid lactation (150 to 200 DIM) were stratified by parity, DIM, and milk production, and were randomly assigned to 1 of 5 treatments arranged in an incomplete crossover design with four 28-d periods. At the end of each period, cows were switched to a different treatment in a way that ensured no cow was on the same treatment in more than 1 period. Cows were housed in a freestall open-sided barn bedded with sand and equipped with Calan gates (American Calan Inc., Northwood, NH) for individual feeding and with misters and fans to minimize heat stress. The following dietary treatments were investigated: (1) a control diet with no sequestering agent or mycotoxin added (**C**); (2) diet C fed with 1,725 µg of AFB₁ (**T**); (3) diet T fed with 20 g/d of sequestering agent 1 (**SEQ1**); (4) diet T fed with 20 g/d of sequestering agent 2 (**SEQ2**); (5) diet T fed with 20 g/d of sequestering agent 3 (**SEQ3**). The sequestering agents were supplied by Diamond V Inc. (Cedar Rapids, IA). They were proprietary formulations of an *S. cerevisiae*

fermentation product containing a low (SEQ1) or high (SEQ2) dose of a proprietary chlorophyll-based additive or a low dose of the additive combined with sodium bentonite clay (SEQ3).

Diets were formulated to meet or exceed the nutrient requirements of cows producing at least 30 kg of milk (NRC, 2001) using CPM-Dairy software version 3.0.10 (www.cpm dairy.net). The ingredients and chemical composition of the basal diet are shown in Table 1. Cows were fed 1,725 µg/head per day of AFB₁ based on an average projected DMI of 23 kg/d, to supply a daily dose of 75 µg/kg of AFB₁ (in treatments T, SEQ1, SEQ2, SEQ3). Dietary AFB₁ was obtained from an *Aspergillus parasiticus* (NRRL-2999) culture at the University of Missouri Diagnostic Laboratory (Columbia, MO). The AFB₁ was mixed with ground corn and molasses, weighed into gelatin capsules and dosed orally to cows once daily before the TMR was fed in the morning; the sequestering agents (SEQ1, SEQ2, SEQ3) were top-dressed on the relevant TMR from d 1 to 28 of each period. The first 20 d of each period were used to ensure adaptation to a new diet and clearance of AFM₁ residues from the milk from the preceding period. Mycotoxin-contaminated corn was fed only during d 21 to 25 of each period in the relevant treatments, and clearance of the toxin from the milk was monitored during d 26 to 28. Queiroz et al. (2012) confirmed the suitability of the 5-d toxin-dosing and 3-d toxin-clearance periods for modeling the effects of sequestering agents on ingested AFB₁.

Experimental Measurements and Analytical Procedures

Cows were milked twice daily. Milk yield was recorded daily by the Afikim milking system (Afikim mini laboratory, Afimilk Ltd., Israel); milk samples were collected from both 1000 and 2200 h milkings on d 20 to 28 in each period and analyzed separately for milk components. Concentrations of milk fat, milk protein, and SCC were analyzed by the Southeast Milk dairy laboratory (Bellevue, FL) using a Bentley 2000 near-infrared reflectance spectrophotometer (Bentley Instruments Inc., Chaska, MN). Values for 3.5% FCM yield were calculated based on the following equation: $[(0.4324 \times \text{milk yield}) + (16.218 \times \text{milk fat yield})]$ (NRC, 2001). Milk AFM₁ concentrations were measured on composited am and pm milk samples on d 20 to 28 in each period and were quantified by the Southeast Milk dairy laboratory using the radioimmunoassay test (CHARM II, Charm Sciences Inc., Malden, MA) described by Diaz et al. (2004). Aflatoxin M₁ excretion was calculated as the product of milk AFM₁ concentration and milk yield. Transfer of the toxin to milk during the

Table 1. Ingredient and chemical composition of the experimental diet

Item	Amount
Ingredient composition (% of DM)	
Corn silage	23.5
Alfalfa hay	6.9
Ryegrass/triticale mixture	8.9
Wet brewers' grain	7.8
Corn grain	23.5
Dried citrus pulp	6.5
Soy hulls	5.9
Whole cottonseed	4.9
Soybean meal (47% CP)	5.9
Amino Plus ¹	2.9
Mineral and vitamin mix ²	3.3
Chemical composition	
Crude protein (%)	16.0
ADF (%)	20.9
NDF (%)	34.2
NE _L (Mcal/kg)	1.66
Calcium (%)	0.86
Phosphorous (%)	0.50
Magnesium (%)	0.70
Potassium (%)	1.31
Sodium (%)	0.38
Iron (mg/kg)	406
Zinc (mg/kg)	110
Copper (mg/kg)	29
Manganese (mg/kg)	98
Aflatoxins (μg/kg)	ND ³
Deoxynivalenol (mg/kg)	ND
T-2 toxin (mg/kg)	ND
Zearalenone (mg/kg)	ND

¹Amino Plus (Ag Processing Inc., Omaha, NE) composition (% DM): lysine 3.5, methionine 0.75, arginine 3.85, tryptophan 0.70, histidine 1.47, leucine 4.0, isoleucine 2.43, phenylalanine 2.64, threonine 2.01, valine 2.37, tyrosine 1.69, choline 0.29.

²Mineral and vitamin mix (% as is): ProVAAL Lys+Met 30 (Perdue Agribusiness, Salisbury, MD), sodium bicarbonate 28.5, DCAD Plus 13.0 (Soychlor), Landus Cooperative, Ralston, IA, calcium carbonate 9.0, dicalcium phosphate 7.0, magnesium oxide 7.0, salt (NaCl) 3.5, Zinpro Availa 4 1.2 (Zinpro Corporation, Eden Prairie, MN), Sel-Plex 1000 0.6 (Alltech, Nicholasville, KY), Rumensin 90 0.22 (Elanco, Eli Lilly, Indianapolis, IN).

³Concentrations of respective toxins were below lower detection limits (5 μg/kg for aflatoxins and 0.5 mg/kg for deoxynivalenol, T-2 toxin, and zearalenone).

dosing period was calculated as daily AFM₁ excretion/daily AFB₁ intake. Transfer of the toxin during the withdrawal period was calculated as daily AFM₁ excretion/AFB₁ intake on d 25. Aflatoxin M₁ clearance rates were calculated by dividing the difference between the AFM₁ concentration on d 26, 27, and 28 and that on d 25 by 24, 48, and 72 h, respectively.

The amount of feed offered at each feeding was recorded for each cow. Refused feed was collected and weighed each morning before feeding and used to calculate intakes for the next day. Diets were fed ad libitum (110% of the previous day's DM intake). In each period, dietary ingredients (corn silage, ryegrass triticale silage mixture, alfalfa orchardgrass hay mixture, and concen-

trate mix) and orts were sampled (100 g) daily into labeled bags from d 20 to 28 and stored in a refrigerator (4°C) for further analysis. Dietary samples were dried at 55°C for 48 h in a forced-air oven, ground to pass the 1 mm screen of a Wiley mill (A. H. Thomas, Philadelphia, PA), and composited across days. Feed samples were sent to the Dairy One forage testing laboratory (Ithaca, NY) for DM, N, ash, NDF, ADF, and mineral analyses. Nitrogen was determined by rapid combustion using a Macro Elemental N analyzer (AOAC International, 2000; Vario MAX CN, model ID 25.00–5003; Elementar, Hanau, Germany) and CP was calculated by multiplying the N value by 6.25. Concentrations of NDF and ADF were measured using the non-sequential method of Van Soest et al. (1991) in an Ankom A200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Heat-stable α-amylase was used in the NDF procedure, and the results are expressed exclusive of residual ash. Minerals (Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn) were analyzed using an inductively coupled plasma radical spectrometer (ICAP, Thermo Scientific Inc., Marietta, OH) after microwave digestion. The concentration of aflatoxins, deoxynivalenol, zearalenone, and T-2 toxin in the feed ingredients was analyzed by HPLC at the University of Missouri Veterinary Diagnostic Laboratory using the HPLC method described by Kutz et al. (2009).

On d 20 and 25 of each period, blood was collected before the morning feeding from the coccygeal vessels into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin anticoagulant, centrifuged at 2,500 × g for 20 min at 4°C and stored at –20°C for further analysis of acute-phase proteins. Plasma haptoglobin concentrations were determined by measuring haptoglobin/hemoglobin complexing based on differences in peroxidase activity (Makimura and Suzuki, 1982). Plasma ceruloplasmin oxidase activity was measured using the colorimetric procedure described by Demetriou et al. (1974). Acid-soluble protein concentration was determined using the bicinchoninic acid kit for protein determination (Sigma-Aldrich, St. Louis, MO) following plasma acid-soluble protein extraction (Nakajima et al., 1982). Whole blood was analyzed for total and differential cell counts using an automated ProCyte Dx hematology analyzer (Idexx Laboratories Inc., Westbrook, ME). The percentage of neutrophils exhibiting phagocytosis and oxidative burst activities after *Escherichia coli* challenge was measured according to procedures described by Martinez et al. (2012). Briefly, neutrophils were loaded with dihydrorhodamine 123 (Sigma-Aldrich Co., St. Louis, MO) and then cocultured with propidium iodide-labeled *E. coli* isolated from the milk of a cow with mastitis at

a bacteria:neutrophil ratio of 40:1. The samples were run within 2 h of preparation using a Becton Dickinson Accuri C6 digital analyzer flow cytometer (Becton Dickinson). Neutrophils were identified according to their size and granularity using forward and side scatter detectors, and data were analyzed using Flowjo software (version 7.6.5., Tree Star Inc., Ashland, OR). The parameters quantified were mean fluorescence intensity (MFI) for propidium iodide staining as a proxy for the number of bacteria phagocytized per neutrophil, and MFI for dihydrorhodamine 123 staining as an indicator of the amount of reactive oxygen species generated per neutrophil. Adhesion molecules that regulate the migration of leukocytes between blood vessel and lymphoid organs, L-selectin (CD62L) and β 2-integrin (CD18), were analyzed as described by Silvestre et al. (2011). Interferon- γ , the proinflammatory cytokine produced by various activated leukocyte populations, was measured as described by Garcia Orellana (2012) using the bovine IFN- γ DuoSet ELISA Development Kit (R&D Systems, Inc., Minneapolis, MN).

Statistical Analysis

The experiment had an incomplete crossover design with 12 experimental units per treatment. Data were analyzed using the GLIMMIX procedure of SAS Institute Inc. (2012). The model for analyzing outcomes measured repeatedly over time on the same experimental unit (milk production and components; DMI; and aflatoxin concentration, transfer, and excretion) included the effects of treatment, period, and day; interactions of treatment and day and day and period; and the random effect of cow. Time was used in the repeated measures statement with autoregressive order 1 [AR(1)] as the time-series covariance structure. We used pre-experimental milk yields as covariates for milk yield data obtained during the predosing (d 16 to 20), toxin-dosing (d 21 to 25), and withdrawal (d 26 to 28) periods. The model for analyzing measures that were not collected serially on the same experimental unit (blood cell counts and immune response data) included the effects of treatment and period and the random effect of cow. Blood sample data from d 20 were used as covariates for analysis of data from samples collected on the last day of toxin-dosing (d 25). We estimated denominator degrees of freedom using the Kenward-Roger option in the MODEL statement for all data, and we corrected for potential carryover effects of treatments between periods by including carryover coefficients in the model (Littell et al., 2006; Jones and Kenward, 2015). We examined data for normality by examining the distribution of residuals and transforming them

as needed before statistical analysis. Post hoc mean comparisons were performed using the Tukey-Kramer procedure for pairwise multiple comparisons. Significance was declared at $P \leq 0.05$, and trends toward significance were declared at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Milk AFM₁ Concentration

In addition to being sufficient to raise the AFM₁ concentration beyond the FDA action level of 0.5 $\mu\text{g/kg}$ for milk, the level of AFB₁ dosed (75 $\mu\text{g/kg}$) exceeded the FDA action level of 20 $\mu\text{g/kg}$ for dairy cattle feeds. However, it represented a moderately high typical natural level of AFB₁ contamination in corn samples for livestock feeds, which can be up to 158 $\mu\text{g/kg}$ (Pietri et al., 2004), even though in other studies (Pietri and Piva, 2007), most of the samples had concentrations of less than 20 $\mu\text{g/kg}$. Doses used in mycotoxin challenge studies usually range from 20 to 112 $\mu\text{g/kg}$ (Diaz et al., 2004; Firmin et al., 2011; Xiong et al., 2015).

The sequestering agent mixtures used in this study were tested to explore whether combining the aflatoxin-binding and immunomodulatory effects of *S. cerevisiae* and the aflatoxin-binding effects of clay and chlorophyll-based products would result in synergistic improvements in binding of aflatoxin, stimulation of the immune response, and enhancement of the performance of dairy cows. The protective effect of *S. cerevisiae* against mycotoxins is due to adhesion of the toxin to the cell wall components (mannan and β -glucan) of the yeast (Pfliegler et al., 2015). Yeast-based products also act as immune modulators because β -glucan can stimulate both nonspecific and specific immunological responses, thus improving the performance of animals (Keller et al., 2015). When fed at levels above 1% of the diet DM to dairy cows (Diaz et al., 2004; Queiroz et al., 2012), clays bind aflatoxins by chemisorption via cation exchange (Phillips et al., 1995). Chlorophyll-based compounds sequester aflatoxin and stimulate the immune response by trapping the toxin via the planar ring structure of the chlorophyll (Arimoto et al., 1993) and stimulating increased activity of glutathione S-transferase, which prevents aflatoxin epoxide from forming an adduct with DNA, facilitating the clearance of the toxin from the body (Hsu et al., 2008). None of the treatments reduced the milk aflatoxin concentration, suggesting that the active ingredient amounts in the sequestering agent mixtures were inadequate for considerable toxin binding. Nevertheless, amounts were sufficient to modulate the immune response. SEQ1 had the greatest proportion of the *S. cerevisiae* fermentation

product; its greater beneficial effect on indices of the immune response highlights the immunomodulatory effects of the *S. cerevisiae* fermentation product. The presence of clay in SEQ3 and not in SEQ2 probably reduced the adverse effects of the toxin on the cows, such that they were able to mount a more pronounced immune response. SEQ2, which had the greatest proportion of the chlorophyll-based additive, had the least beneficial effect on immune response, suggesting that the additive may have been less effective at binding the aflatoxin or modulating the immune response than the *S. cerevisiae* fermentation product or the clay.

We detected no AFM₁ in the milk of the cows before they were dosed with AFB₁ (data not shown). During the toxin-dosing period, all cows except for those fed diet C received 1,725 µg of AFB₁ daily, and their DMI averaged 23.6 kg/d; the average daily ingestion of AFB₁ was 73 µg/kg of DMI. We detected no AFM₁ in the milk of cows fed diet C, whereas cows fed diets T, SEQ1, SEQ2, and SEQ3 had mean milk AFM₁ concentrations of 0.73, 0.74, 0.73, and 0.74 µg/kg, respectively (Table 2). Adding sequestering agents to diets containing the toxin did not affect milk AFM₁ concentration, excretion, or transfer during the toxin-dosing period. Therefore, none of the sequestering agent treatments prevented milk AFM₁ concentrations from rising beyond the FDA action level (0.5 µg/kg). This is consistent with other studies on the AFB₁-sequestering ability of yeast cell

wall products that contain high levels of AFB₁ contamination. Milk AFM₁ concentration was unaffected by adding 10 g/d of an undisclosed yeast cell wall product or 125 g/d of a yeast cell wall-based product to dairy cow diets containing 100 µg/kg (Waltman et al., 2008) or 112 µg/kg (Kutz et al., 2009) of AFB₁, respectively. Likewise, Kissell et al. (2013) showed that yeast oligosaccharide-based additives did not affect the milk AFM₁ concentration of cows fed diets containing 80 µg/kg AFB₁. In addition, Firmin et al. (2011) reported that adding an undisclosed yeast cell wall product at 2 g/kg of the diet did not affect the concentration of AFM₁ in the milk of dairy ewes fed a diet containing 60 µg of AFB₁/kg. However, yeast cell wall sequestering agents have effectively sequestered AFB₁ when the toxin concentration in the diet was lower (Diaz et al., 2004; Xiong et al., 2015). Diaz et al. (2004) reported that adding a yeast cell wall-based product at 10 g/d to a dairy cow diet contaminated with 55 µg/kg of AFB₁ reduced the milk AFM₁ concentration. Certain combinations of yeast- and clay-based products have also been less effective at high contamination levels. Xiong et al. (2015) reported that adding a blend of yeast extract and yeast culture, hydrated calcium sodium aluminosilicates, and other metabolites at 42.5 g/kg of DMI reduced milk AFM₁ concentration, secretion, and transfer in diets contaminated with 20 µg/kg of AFB₁ but had no effect in diets contaminated with 40

Table 2. Effect of dosing aflatoxin B₁ (AFB₁) with or without sequestering agents¹ on the concentration, transfer, and excretion of aflatoxin M₁ in the milk of dairy cows during the toxin-dosing and withdrawal periods

Item	Treatment ²				SEM	P-value
	T	SEQ1	SEQ2	SEQ3		
Toxin-dosing period (d 21–25)						
Concentration (µg/kg)	0.73	0.74	0.73	0.74	0.01	0.74
Excretion (µg/d)	19.45	19.62	19.15	16.68	1.26	0.88
Transfer (%)	1.13	1.14	1.11	1.08	0.07	0.88
Withdrawal period: first day (d 26)						
Concentration (µg/kg)	0.63 ^{a,x}	0.43 ^{b,y}	0.46 ^{ab,y}	0.42 ^{b,y}	0.06	0.01
Excretion (µg/d)	15.87 ^x	11.14 ^y	12.78 ^{xy}	11.23 ^y	1.99	0.09
Transfer (%)	0.92 ^x	0.65 ^y	0.74 ^{xy}	0.65 ^y	0.12	0.09
Clearance (µg/h)	0.01	0.01	0.01	0.01	0.00	0.85
Withdrawal period: second day (d 27)						
Concentration (µg/kg)	0.06	0.04	0.05	0.03	0.03	0.76
Excretion (µg/d)	1.25	1.02	1.75	1.06	0.82	0.79
Transfer (%)	0.07	0.06	0.10	0.06	0.04	0.79
Clearance (µg/h)	0.01	0.01	0.01	0.01	0.00	0.88
Withdrawal period: third day (d 28)						
Concentration (µg/kg)	ND ³	ND	ND	ND		
Clearance (µg/h)	0.01	0.01	0.01	0.01	0.00	0.88

^{a,b}Means within a row with no common superscripts differ ($P \leq 0.05$).

^{x,y}Means within a row with no common superscripts tend to differ ($P \leq 0.10$).

¹Proprietary *Saccharomyces cerevisiae* fermentation product-based sequestering agents (Diamond V Inc., Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

²T = control diet + AFB₁; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.

³ND = not detected.

$\mu\text{g/kg}$ of AFB₁. In general, the efficacy of clay or yeast cell wall products alone or in combination depends on the dose of the sequestering agent and the level of mycotoxin contamination in the diet.

Once AFB₁ was withdrawn from the diet, milk AFM₁ concentrations in cows fed the sequestering agents decreased rapidly, such that they fell below the FDA action level within 24 h (Table 2), whereas it took 48 h for milk AFM₁ concentrations to decrease below the FDA action level in cows fed diet T. In particular, milk AFM₁ concentrations in cows fed SEQ1 and SEQ3 were lower ($P < 0.05$) than those of cows fed T 24 h after withdrawing the toxin (Figure 1). This was because the SEQ1 and SEQ3 diets tended to reduce ($P \leq 0.10$) the transfer and excretion of the toxin from d 25 into the milk on d 26. The SEQ2 diet tended to reduce ($P = 0.06$) the milk AFM₁ concentration compared with that for cows fed diet T, but it had no effect on the transfer and excretion of the toxin into the milk. Our previous study (Queiroz et al., 2012) showed that during the toxin-dosing period, a diet containing a montmorillonite clay adsorbent (1% of the diet DM) reduced the milk AFM₁ concentration below the FDA action level when cows were fed a diet contaminated with 75 $\mu\text{g/kg}$ DMI of AFB₁. However, the adsorbent had no effect when it was fed at 0.2% of the diet DM. The dose of clay fed in the current study (0.04% of DMI) was lower

than in our previous study, but when combined with the *S. cerevisiae* fermentation product, it reduced the time required for a reduction in milk AFM₁ concentration to a safe level by 24 h after dietary withdrawal of the toxin.

By 48 h after withdrawing AFB₁ from the diet, only trace concentrations of AFM₁ were detected in the milk in all treatments and no treatment effects ($P > 0.10$) on the concentration, transfer, or excretion of the toxin were evident. This is consistent with other studies, which reported that AFM₁ concentrations in milk were reduced drastically within 2 to 3 d of withdrawing AFB₁ from the diet (Kutz et al., 2009; Queiroz et al., 2012; Xiong et al., 2015). Clearance rates of milk AFM₁ did not differ among cows fed the toxin during any of the days of the withdrawal period.

Animal Performance

During the predosing period, milk yield and DMI did not differ across treatments (26.7 ± 1.20 , $P = 0.64$ and 22.6 ± 0.81 , $P = 0.30$ kg/d, respectively; Table 3). Therefore, feeding the sequestering agents during the adaptation or predosing periods did not affect the performance of the cows. During the toxin-dosing period, DMI was not affected by dietary treatment (23.7 ± 0.85 kg/d, $P = 0.16$). Also, dietary treatments did

Figure 1. Effect of dosing aflatoxin B₁ (AFB₁) with or without sequestering agents on the concentration of aflatoxin M₁ (AFM₁) in the milk of dairy cows. Toxin-dosing period = d 21 to 25, withdrawal period = d 26 to 28. *AFM₁ concentrations on the day specified differed ($P = 0.01$). Error bars indicate standard errors. SEQ1, SEQ2, and SEQ3 are proprietary *Saccharomyces cerevisiae* fermentation product-based sequestering agents (Diamond V Inc., Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3); T = control diet + AFB₁; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3. Color version available online.

Table 3. Effect of dosing aflatoxin B₁ (AFB₁) with or without sequestering agents¹ on the performance of cows during the predosing (d 16 to 20), toxin-dosing (d 21 to 25), and withdrawal (d 26 to 28) periods

Item	Treatment ²					SEM	P-value
	C	T	SEQ1	SEQ2	SEQ3		
Predosing period							
DMI (kg/d)	23.4	22.7	22.4	22.7	21.9	0.81	0.30
Milk yield (kg/d)	27.6	26.6	26.5	26.7	26.1	1.20	0.64
Toxin-dosing period							
DMI (kg/d)	24.4	24.2	23.7	23.0	23.1	0.85	0.16
Milk yield (kg/d)	28.5 ^{a,x}	26.0 ^{ab,y}	26.7 ^{ab,xy}	25.9 ^{ab,y}	24.8 ^{b,y}	1.34	0.01
Milk yield change ³ (kg/d)	0.8	-0.7	0.1	-0.7	-1.1	0.82	0.16
3.5% FCM (kg/d)	29.8 ^{a,x}	27.1 ^{ab,y}	27.7 ^{ab,xy}	26.9 ^{b,y}	25.9 ^{b,y}	1.52	0.01
Efficiency (3.5% FCM/kg of DMI)	1.21	1.12	1.19	1.25	1.17	0.07	0.37
Milk fat (%)	3.79	3.83	3.80	3.79	3.85	0.07	0.63
Milk fat (kg/d)	1.07 ^{a,x}	0.99 ^{ab,y}	1.00 ^{ab,xy}	1.00 ^{ab,xy}	0.95 ^{b,y}	0.06	0.01
Milk protein (%)	3.23	3.24	3.24	3.26	3.24	0.02	0.64
Milk protein (kg/d)	0.92 ^a	0.84 ^{ab}	0.85 ^{ab}	0.86 ^{ab}	0.79 ^b	0.05	0.01
SCC ($\times$ 1,000/mL)	221	169	209	283	237	59.6	0.34
Withdrawal period							
DMI (kg/d)	24.4	23.5	24.4	24.0	23.2	0.92	0.64
Milk yield (kg/d)	26.2	23.5	25.5	23.9	23.1	1.63	0.21
3.5% FCM (kg/d)	27.3	24.5	26.4	24.7	24.2	1.69	0.17
Efficiency (3.5% FCM/kg of DMI)	1.03	1.00	1.02	0.96	0.99	0.07	0.89
Milk fat (%)	3.88	3.84	3.77	3.77	3.82	0.09	0.60
Milk fat (kg/d)	1.05	0.95	0.99	0.94	0.94	0.06	0.23
Milk protein (%)	3.23 ^{ab}	3.26 ^{ab}	3.30 ^a	3.21 ^b	3.22 ^{ab}	0.03	0.04
Milk protein (kg/d)	0.88	0.81	0.86	0.80	0.79	0.05	0.20

^{a,b}Means within a row with no common superscripts differ ($P \leq 0.05$).

^{x,y}Means within a row with no common superscripts tend to differ ($P \leq 0.10$).

¹Proprietary *Saccharomyces cerevisiae* fermentation product-based sequestering agents (Diamond V Inc., Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

²T = control diet (C) + AFB₁; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.

³Calculated as mean milk yield during the toxin-dosing period – mean milk yield during the predosing period.

not affect the change in milk yield from the predosing to the toxin-dosing periods. However, feeding diet T instead of C during the toxin-dosing period tended to reduce milk yield (26.0 vs. 28.5 kg/d, $P = 0.08$) and 3.5% FCM yield (27.1 vs. 29.8 kg/d, $P = 0.09$). This was consistent with the study of Queiroz et al. (2012), who reported that feeding AFB₁ at the rate used in this study tended to reduce the 3.5% FCM yield of cows. A similar numerical trend was reported when 112 μ g/kg of AFB₁ was fed to dairy cows by Kutz et al. (2009). However, studies have also reported no changes in milk yield due to feeding 117 μ g/kg of DMI (Maki et al., 2016) or 80 μ g of AFB₁/kg of DMI (Kissell et al., 2013).

Feeding the SEQ3 diet instead of C reduced milk yield (24.8 vs. 28.5 kg/d, $P = 0.01$) and 3.5% FCM yield (25.9 vs. 29.8 kg/d, $P = 0.01$). Also, feeding the SEQ2 diet reduced 3.5% FCM yield relative to feeding diet C (26.9 vs. 29.8 kg/d, $P = 0.04$). The reasons for these reductions are not clear. In contrast, the milk and FCM yields of cows fed the SEQ1 and C diets did not differ ($P > 0.10$). Therefore, unlike the other sequestering agents, feeding the SEQ1 diet probably prevented the adverse effect of the toxin on milk production. This

may be due to the numerically higher DMI for cows fed the SEQ1 diet relative to those fed SEQ2 and SEQ3. Feed efficiency was unaffected ($P > 0.10$) by dietary treatments.

Compared with diet C, diet T tended to reduce milk fat yield ($P = 0.10$) and diet SEQ3 reduced milk fat yield ($P = 0.01$) and milk protein yield ($P = 0.01$; Table 3). These results reflect the reduction in milk yields caused by feeding the T and SEQ3 diets, because milk fat and protein concentrations were not influenced by dietary treatment. The latter agrees with Smith et al. (1994), who did not detect changes in milk protein and fat percentages due to feeding 200 μ g of AFB₁/kg of diet DM to goats. In addition, Kutz et al. (2009) reported that milk protein and fat percentages were unaffected when dairy cows were fed 112 μ g of AFB₁/kg of diet DM.

No dietary treatment effects on performance measures were evident during the withdrawal period, except that the milk protein concentration of cows fed the SEQ1 diet was greater ($P = 0.04$) than those of cows fed the SEQ2 diet, but none of the values differed from those of cows fed diet C (Table 3).

Table 4. Effect of dosing aflatoxin B₁ (AFB₁) with or without sequestering agents¹ on blood cell counts of dairy cows

Item	Treatment ²					SEM	P-value
	C	T	SEQ1	SEQ2	SEQ3		
White blood cells (10 ³ /μL)	15.22	14.99	14.85	15.14	14.65	0.52	0.94
Red blood cells (10 ⁶ /μL)	6.08 ^a	5.80 ^b	5.87 ^{ab}	5.93 ^{ab}	5.85 ^{ab}	0.07	0.05
Neutrophils (10 ³ /μL)	3.26	3.43	3.28	3.26	3.36	0.36	0.98
Monocytes (10 ³ /μL)	1.65	2.11	1.49	1.87	1.65	0.28	0.22
Eosinophils (10 ³ /μL)	0.37	0.38	0.40	0.40	0.40	0.03	0.93
Lymphocytes (10 ³ /μL)	9.51	8.93	10.55	8.81	9.58	0.68	0.12
Basophils (10 ³ /μL)	0.11	0.05	0.02	0.02	0.00	0.03	0.12
Hemoglobin (g/dL)	9.97 ^a	9.48 ^b	9.65 ^{ab}	9.62 ^{ab}	9.57 ^{ab}	0.14	0.02
Hematocrit (%)	29.85	28.59	28.85	29.06	28.78	0.52	0.16

^{a,b}Means within a row with no common superscript differ ($P \leq 0.05$).

¹Proprietary *Saccharomyces cerevisiae* fermentation product-based sequestering agents (Diamond V Inc., Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

²T = control diet (C) + AFB₁; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.

Blood Parameters

Compared with diet C, feeding diet T reduced red blood cell count ($P = 0.05$) and hemoglobin concentration ($P = 0.02$; Table 4). However, blood cell counts were within the normal range for healthy adult dairy Holstein cows in mid lactation (George et al., 2010). We found no previous studies on the effect of dietary AFB₁ on the blood cell counts of dairy cows, but studies in poultry (Huff et al., 1986; Mohiuddin et al., 1986; Basmacioglu et al., 2005) and rams (Dönmez et al., 2012) have shown that aflatoxin suppressed blood cell counts.

Expression of CD62L and CD18 tended to be highest in cows fed the SEQ1 ($P = 0.10$) and SEQ3 ($P = 0.09$) diets (Table 5). Blood neutrophils act as the first line of defense against many pathogens, and migration of neutrophils involves adhesion mediated by selectins (Silvestre et al., 2011). After this initial phase, the neutrophil is activated for adhesion of its integrins to the vascular endothelium (Silvestre et al., 2011). The greater MFI of staining for CD62L and CD18 on neutrophils of cows fed the SEQ1 and SEQ3 diets suggests that these agents altered the migration of neutrophils exposed to the toxin. Dietary treatments did not affect the production of IFN- γ and or the percentage of cells exhibiting phagocytosis and oxidative burst activities (Table 5).

The production of proinflammatory cytokines is promoted by inflammasomes, which are a specialized intracellular multiprotein complex (Schroder and Tschoop, 2010). These are innate immune system receptors that trigger inflammation in response to infectious microbes, toxins, or both (Guo et al., 2015). Inflammasomes are activated by the lethal anthrax toxin

(Boyden and Dietrich, 2006), bacterial toxins such as Shiga toxin (Lee et al., 2015), and bacterial and viral pathogens (Guo et al., 2015). To our knowledge, the role of inflammasomes in mycotoxin-mediated inflammation is unknown. Future studies should examine the involvement of inflammasomes in mycotoxin-mediated responses in dairy cows.

Acute-phase proteins are blood proteins that play major roles in systemic reaction to inflammation (Cecilian et al., 2012), and their increased secretion is an indication of inflammatory stress (Bertoni et al., 2008; Gonzalez et al., 2008; Cecilian et al., 2012). Haptoglobin and ceruloplasmin concentrations were not affected by dietary treatments (Table 5). However, acid-soluble protein concentrations were greater with diet T than with SEQ2 ($P = 0.01$), and tended to be greater with T than with SEQ1 ($P = 0.08$) and SEQ3 ($P = 0.10$). Increases of acid-soluble protein concentration in response to inflammatory stress have been reported in goats (Gonzalez et al., 2008), sheep (Eckersall et al., 2007), pigs (Eckersall et al., 1996), and cows (Nagahata et al., 1989). The result in this study suggest that feeding the SEQ2 diet reduced the inflammatory response caused by diet T, and the SEQ1 and SEQ3 diets tended to have a similar effect. Queiroz et al. (2012) fed the same dose (75 $\mu\text{g/kg}$) of AFB₁ as that in the current study to dairy cows and reported that haptoglobin and β -integrin concentrations were increased by the toxin. However, Xiong et al. (2015) reported that adding 20 or 40 $\mu\text{g/kg}$ of AFB₁ to the diet of dairy cows had no effect on the plasma concentrations of antibodies, IgG, IgM, and IgA. The latter study may have been less successful at detecting the effects of AFB₁ on measures of the immune response because they fed lower doses of AFB₁ and examined different immune-response indi-

Table 5. Effect of dosing aflatoxin B₁ (AFB₁) with or without sequestering agents¹ on immune response indices of dairy cows

Item	Treatment ²					SEM	P-value
	C	T	SEQ1	SEQ2	SEQ3		
Granulocyte adhesion molecules							
β ₂ -Integrin (CD18; MFI ³)	918 ^y	908 ^y	1,278 ^x	956 ^y	1,312 ^x	202	0.10
L-Selectin (CD62L; MFI)	2,188 ^y	2,353 ^y	3,284 ^x	2,270 ^y	3,115 ^x	516	0.09
Neutrophil function							
Neutrophil phagocytosis (% of cells)	27.72	26.55	28.27	27.69	28.45	2.28	0.94
Oxidative burst (% of cells)	2.94	2.08	3.76	3.06	1.61	0.78	0.11
Cytokine ⁴							
Interferon-γ (MFI)	4.79	6.56	6.75	4.55	6.16	1.52	0.46
Interferon-γ (% of cells)	3.27	3.08	3.48	3.90	2.43	0.82	0.46
Acute-phase proteins							
Haptoglobin ⁵	0.03	0.02	0.03	0.02	0.08	0.03	0.44
Ceruloplasmin (mg/100 mL)	19.50	20.59	20.79	20.57	20.28	1.01	0.72
Acid-soluble protein (mg/100 mL)	4.39 ^{ab,xy}	5.10 ^{a,x}	4.25 ^{ab,xy}	3.69 ^{b,y}	4.15 ^{ab,y}	0.35	0.01

^{a,b}Means within a row with no common superscript differ ($P \leq 0.05$).

^{x,y}Means within a row with no common superscript tend to differ ($P \leq 0.10$).

¹Proprietary *Saccharomyces cerevisiae* fermentation product-based sequestering agents (Diamond V Inc., Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

²T = control diet (C) + AFB₁; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.

³MFI = mean fluorescence intensity.

⁴Fold increase of ionomycin and phytohemagglutinin-stimulated cells over the nonstimulated cells.

⁵Arbitrary unit based on absorbance at 450 nm.

ces. This study showed that the 3 sequestering agents altered the immune status of cows challenged with the toxin and reduced toxin-induced inflammatory stress.

CONCLUSIONS

Feeding 75 µg/kg of AFB₁ increased AFM₁ concentration in the milk of lactating dairy cows beyond the FDA action level of 0.5 µg/kg. Feeding the sequestering agents with the toxin did not prevent the milk AFM₁ concentration from exceeding the FDA action level. However, within 24 h of withdrawing the toxin, diets that included the sequestering agents had reduced the milk AFM₁ concentration below the FDA action level and diet T had not. Feeding AFB₁ tended to reduce the yields of milk, 3.5% FCM, milk fat, and milk protein and reduced red blood cell count and hemoglobin concentration. Feeding the SEQ1 diet prevented the reduction in milk and 3.5% FCM yield caused by the toxin and increased the MFI of CD62L and CD18 relative to those for diets T and C. Feeding SEQ2 or SEQ3 did not prevent the adverse effect of the toxin on milk yield or 3.5% FCM yield. However, the SEQ2 diet reduced the acid-soluble protein concentration relative to that for cows fed diet T, and the SEQ3 diet increased the MFI of CD62L and CD18 relative to those for cows fed diets SEQ2, T, or C. These results imply that the sequestering agents were not effective at preventing the toxin from being transferred to the milk, but they did reduce toxin-induced inflammatory stress in cows. In addition,

SEQ1 reduced the adverse effects of the toxin on milk production. Future studies should validate the results of the current study and elucidate the relative efficacy and synergistic effects of bentonite clays, *S. cerevisiae* fermentation products, cell wall oligosaccharides, and chlorophyll-based additives at alleviating the toxic effects of aflatoxin on dairy cows.

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