Feed additives containing sequestrant clay minerals and inactivated yeast reduce aflatoxin excretion in milk of dairy cows

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ABSTRACT

The objective was to evaluate the efficacy of 2 dietary mycotoxin sequestrants, Toxy-Nil (TN) or Unike Plus (UP), in reducing aflatoxin (AF) M₁ concentrations in milk of dairy cows challenged with dietary AF. Thirty-two mid-lactation Holstein cows were blocked by parity, days in milk, and milk yield and were randomly assigned within block to receive one of the following treatments: (1) 2.8 mg of AF/cow per d (positive control, PC), (2) 2.8 mg of AF + 100 g of TN/cow per d, (3) 2.8 mg of AF + 100 g of UP/cow per d, or (4) no AF and no additives (negative control, NC). For 7 d, treatments, dispersed in 150 g of sweet feed carrier, were top-dressed twice daily by mixing into the top portion of the TMR at each feeding. After the experimental period, cows were fed the NC diet and clearance of AFM₁ via milk was monitored for 7 d. Feed and water were available ad libitum throughout the trial. Treatments had no effect on feed intake, milk yield, milk composition, or milk somatic cell count. Relative intake of AF was similar among PC, TN, and UP, averaging 106.5, 107.6, and 102.5 ± 2.9 µg/kg of diet dry matter, respectively. Relative intake of mycotoxin sequestrants was similar between TN and UP, averaging 0.4 and 0.4 ± 0.1% of diet dry matter, respectively. Concentration and mass of AFM₁ secreted in milk and in urine were similar between TN and UP, but were lower than PC; concentrations in milk averaged 0.2, 0.3, and 0.6 ± 0.1 µg/kg, respectively, and mass secreted in milk averaged 8.1, 9.8, and 20.5 ± 1.7 µg/d. Concentrations in urine averaged 6.9, 7.4, and 14.2 ± 1.5 µg/L, respectively, and mass secreted in urine averaged 225.7, 250.8, and 521.6 ± 53.1 µg/d. Likewise, concentration and mass of free AF excreted in feces were similar between TN

and UP, but were lower than PC; concentrations averaged 7.7, 8.9, and 12.4 ± 0.6 µg/kg, respectively, and mass excreted averaged 57.8, 69.6, and 95.6 ± 4.8 µg/d. Transfer of AF from feed to AFM₁ in milk was reduced by 63 and 52%, and in urine, by 57 and 52% for TN and UP, respectively. Transfer of AF from feed to free AF in feces was reduced by 38 and 26% for TN and UP, respectively. The clearance rate of AFM₁ in milk did not differ among PC, TN, and UP (46.1, 66.5, and 50.0 ± 6.7%/d, respectively). Results indicate that dietary inclusion of 100 g of TN or UP significantly reduced AFM₁ in milk of cows consuming TMR containing approximately 105 µg of AF/kg of diet dry matter. Results also suggest that both TN and UP reduced absorption of AF.

Key words: adsorbent, aflatoxin M₁, mycotoxin

INTRODUCTION

Aflatoxins (AF) are mycotoxins produced by several species of fungi. Of particular interest in livestock feed are those of the genus *Aspergillus*, present in 20 to 30% of feeds globally (Streit et al., 2013), and estimated to exceed \$250 million in annual costs to crop and livestock operations in the United States alone (Richard et al., 2003). Aflatoxin B₁ is the major AF produced and it is considered the most toxic naturally occurring toxin (Squire, 1981; Bennett and Klich, 2003). It is quickly absorbed in the upper gastrointestinal tract of animals due to its low molecular weight (312 Da; Yiannikouris and Jouany, 2002; Moschini et al., 2007; Gallo et al., 2008), being detected in plasma of dairy animals by 5 min after ingestion (Gallo et al., 2008). After absorption, AFB₁ is metabolized in the liver by the cytochrome P450 system, forming AFM₁ (Kuilman et al., 2000), a hepatotoxin and immune suppressor (Wogan, 1999; Jiang et al., 2005) classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2002). Aflatoxin M₁ has been detected in milk and urine by 6 h after ingestion of AFB₁ (Helferich et al., 1986; Battacone et al., 2003).

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Challenging dairy cows with dietary AFB₁ has been shown to reduce milk yield (Applebaum et al., 1982), milk protein concentration, and milk fat yield (Queiroz et al., 2012). Furthermore, AFB₁ diminished cellular and humoral immune responses of toxin-fed pigs (Marin et al., 2002) and increased incidence of fatty liver and degeneration and necrosis of liver cells in rats (Abdel-Wahhab et al., 2002). In the United States, the Food and Drug Administration (FDA) has set limits for AF in feed (20 µg/kg) and for AFM₁ in milk (0.5 µg/kg); in Europe, milk concentrations of AFM₁ may not exceed 0.05 µg/kg (FDA, 2000; EFSA, 2004).

Various compounds with adsorbent characteristics have been evaluated as potential dietary additives to reduce absorption of AF. Examples of such toxin-binding compounds include bentonite, kaolin, montmorillonite clays, hydrated sodium calcium aluminosilicate, and glucomannan (Ramos and Hernández, 1997; Abdel-Wahhab et al., 2002; Diaz et al., 2002; Queiroz et al., 2012; Carraro et al., 2014). These adsorbents bind to dietary AFB₁, making it unavailable for absorption, and thereby reducing formation of AFM₁ and its secretion into milk (Moschini et al., 2008; Gallo and Masoero, 2010; Queiroz et al., 2012). The ability of these compounds to reduce AFM₁ in milk is related both to level of inclusion of the products in the diet and to the concentration of AF that is supplied to the cows (Diaz et al., 2004; Kutz et al., 2009; Ogunade et al., 2016). Moreover, there are indications of effects of AFB₁ or the adsorbents, themselves, on milk production, rumen fermentation, and immune status, but responses have been inconsistent. For example, supplementation of an AF adsorbent to dairy cows challenged with AF did not affect mammary, liver, or immune function, but altered rumen characteristics in the presence or absence of dietary AF (Xiong et al., 2015). In contrast, feeding AF tended to reduce milk fat yield and alter some indicators of immune status, but effects of supplemental adsorbents on those variables and AFM₁ concentrations in milk were inconsistent (Queiroz et al., 2012; Ogunade et al., 2016).

Thus, different studies have reported various levels of efficacy, depending on AF dosages and adsorbent compounds tested, and several studies have emphasized the need for further experimentation. We hypothesized that dietary supplementation of lactating dairy cows fed an AF-contaminated diet with either of 2 different mycotoxin sequestrants would reduce AFM₁ concentrations in milk and urine, without affecting milk production. Our objective was to establish and compare the efficacy of 2 dietary mycotoxin sequestrants, TN or UP, on reducing transfer of AF from diet to milk and on production of lactating dairy cows challenged with an AF-contaminated diet.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

The experiment and use of animals was approved by the University of Missouri Animal Care and Use Committee (protocol number 8706). Thirty-two mid-lactation Holstein cows (35.7 ± 4.5 kg/d of milk, 183 ± 70 DIM, and 2.1 ± 0.7 lactations; mean $\pm$ SD) housed in a free-stall facility at University of Missouri Foremost Dairy Research Center were blocked based on DIM, milk yield, and parity. The experiment was conducted as a complete randomized block design, with cow as the experimental unit. Cows within block were randomly assigned to receive 1 of 4 treatments: (1) 2.8 mg of AF (positive control, **PC**), (2) 2.8 mg of AF + 100 g of Toxy-Nil (**TN**; Nutriad Animal Feed Additives, Dendermonde, Belgium), (3) 2.8 mg of AF + 100 g of Unike Plus (**UP**; Nutriad), or (4) no AF and no additives (negative control, **NC**). Dietary AF was supplied as *Aspergillus parasiticus* (NRRL-2999) culture material containing 650 mg/kg of AFB₁, 22 mg/kg of AFB₂, 223 mg/kg of AFG₁, and 5.7 mg/kg of AFG₂. Toxy-Nil is composed of high adsorbent clay minerals and inactivated yeast (*Saccharomyces cerevisiae*), and Unike Plus is composed of high adsorbent clay minerals, inactivated yeast (*S. cerevisiae*), botanical components, and a mixture of antioxidants and preservatives. Treatments were dispersed into 150 g of sweet feed carrier (Non-Medicated Calf Grower, MFA Agri-Services, Columbia, MO) and top-dressed by mixing into the top portion of the TMR at each twice-daily feeding.

The trial consisted of three 7-d periods, which included an initial period for adaptation to grouping, pen housing, and feeding system (d -6 to 0); an experimental period, during which AF and mycotoxin sequestrants were fed to cows as per treatment design (d 1 to 7); and a recovery period, during which all cows received the NC diet and were monitored until levels of AFM₁ in milk were undetectable (d 8 to 14).

Cow Management

The diet was a TMR formulated to meet or exceed predicted nutrient requirements (NRC, 2001). Cows were individually fed twice daily (1530 and 0730 h) using feed bins (American Calan, Northwood, NH). Feed was available ad libitum to about 10% refusals, and intake was recorded. Feeds were sampled every 3 d and DM content was determined by forced-air oven drying at 55°C for 72 h. Diets were adjusted weekly for DM content of ingredients. Feed samples were ground through a 2-mm sieve, combined into a single composite, and analyzed by wet chemistry for chemi-

cal composition (DairyOne Laboratories, Ithaca, NY). The ingredient and chemical composition of the diets is shown in Table 1. Water was available ad libitum.

Cows were milked twice daily (1900 and 0700 h) and milk production was automatically recorded (GEA Group AG, Düsseldorf, Germany). Milk samples for composition were collected on d 0, 7, and 14 of the trial from consecutive p.m. and a.m. milkings, then immediately refrigerated at 4°C with a preservative (Broad Spectrum Microtab, Advanced Instruments, Norwood, MA) until individually analyzed for fat, protein, lactose, SNF, MUN, and SCC by a certified DHIA laboratory (Mid-South Dairy Records, Springfield, MO). Milk composition for each experimental period was calculated from the composite of each milking corrected for milk yield. Energy-corrected milk and 3.5% FCM were calculated as described by Dairy Records Management Systems (2014): ECM (kg/d) = (milk yield, kg/d × 0.327) + (milk fat, kg/d × 12.95) + (milk true protein, kg/d × 7.65), and 3.5% FCM (kg/d) = (milk yield, kg/d × 0.432) + (milk fat, kg/d × 16.216). Feed efficiency was calculated as feed efficiency = milk yield, ECM, or 3.5% FCM, kg/d ÷ DMI, kg/d. Lastly, milk flow rate was calculated as daily milk yield divided by the combined duration of p.m. and a.m. milkings.

On d 0, 7, and 14, BW and rectal temperature were recorded immediately after the morning milking and before the morning feeding. Also, cows were independently assessed by 2 individuals for BCS and locomotion score as described by Wildman et al. (1982) and Sprecher et al. (1997), respectively, and the average of scores was used in the analyses.

Laboratory Analysis of Milk Components. Milk fat, true protein, and lactose were determined by infrared spectroscopy (Bentley 2000, Bentley Instruments, Chaska, MN), MUN by a modified Berthelot reaction (ChemSPec 150, Bentley Instruments), and SCC by flow cytometry (Somacount 300, Bentley Instruments) in a commercial Dairy Herd Improvement Association laboratory (Midwest Dairy Records, Springfield, MO).

Calculation of Energy Balance. Energy balance (EB) was estimated on d 0, 7, and 14 according to prediction equations from NRC (2001). Daily net energy intake (NE_I) from the diet was calculated as NE_I = energy content of the diet × DMI; NE_M was calculated as $NE_M = BW^{0.75} \times 0.08$ for multiparous cows and $NE_M = (0.96 \times BW)^{0.75} \times (0.1 \times BCS + 0.65)$ for primiparous cows; NE_L was calculated as NE_L = milk yield, kg/d × [(0.0929 × fat %) + (0.0574 × true protein %) + (0.0395 × lactose %)]; net energy for pregnancy (NE_P) was calculated as $NE_P = (0.00318 \times \text{days pregnant} - 0.0352) \times (\text{calf BW}/45) \times 4.57$; NE_G was calculated only for first or second lactation cows

using the equation $NE_G = 0.0427 \times BW^{0.75} \times (\text{shrunk weight gain})^{1.097}$, using values for shrunk weight gain of 0.148 kg/d for first lactation and 0.118 kg/d for second

Table 1. Ingredient and chemical composition of the basal diet¹

Item	Amount
Ingredient, % of DM	
Corn silage	35.4
Alfalfa haylage	7.1
Alfalfa hay	7.9
Corn, ground	24.6
Soybean hulls, ground	4.1
Brewer's grains, wet	4.9
Soybean meal, 47.5% CP	6.3
Amino Plus ²	3.4
Blood meal	1.0
Salt, white	0.3
CaCO ₃	1.1
K ₂ CO ₃	0.3
MgO	0.2
NaHCO ₃	0.8
Dynamate ³	0.3
Trace mineral premix ⁴	0.1
Vitamin A, D, E premix	0.1
Vitamin E 20,000	0.1
Rumensin 90 ⁵	0.01
Energy Booster 100 ⁶	1.9
MetaSmart Dry ⁷	0.2
Chemical composition, ⁸ % of DM unless otherwise indicated	
DM, %	45.6
CP	18.4
Available protein	17.5
Soluble protein, % of CP	27.0
ADF	22.2
NDF	31.9
Lignin	3.9
NFC	37.2
Starch	24.9
ESC, ⁹ simple sugars	1.9
Crude fat	4.7
Calcium	1.1
Phosphorus	0.4
Magnesium	0.3
Potassium	1.4
Sodium	0.3
NE _L , Mcal/kg	1.7
DCAD, mEq/100 g	18.0
Total aflatoxin, ¹⁰ µg/kg of DM	ND ¹¹

¹Values represent averages of weekly samples.

²Ag Processing (Emmetsburg, IA).

³The Mosaic Co. (Plymouth, MN).

⁴The trace mineral premix contained 99.85% ground corn, 0.04% CoSO₄, 0.05% ethylenediamine dihydriodide, and 0.06% sodium selenite.

⁵Elanco Animal Health (Indianapolis, IN).

⁶Milk Specialties Global (Eden Prairie, MN).

⁷Adisseo (Antony, France).

⁸Values, provided by DairyOne Laboratories (Ithaca, NY), represent averages of samples composited weekly.

⁹ESC = ethanol-soluble carbohydrates.

¹⁰Total aflatoxin, µg/kg of DM = aflatoxins B₁ + B₂ + G₁ + G₂, µg/kg of DM.

¹¹ND = not detected.

lactation, respectively (Van Amburgh et al., 1998). Estimated EB was calculated by $EB = NE_I - NE_M + NE_L + NE_P + NE_G$.

Aflatoxin Sampling

Milk samples were collected into 50-mL conical tubes during the p.m. and a.m. milkings on d -2, -1, 6, 7, 8, 9, 10, 11, 12, 13, and 14. Afternoon milk samples were stored at 4°C until collection of morning samples, and then samples were composited on a daily basis corrected for milk yield and stored at -20°C until analysis. On d 7, the final day of the experimental period, cows were stimulated to urinate by massaging the area underneath the vulva, and urine was collected into 50-mL conical tubes and stored at -20°C. Additionally, feces were collected from the rectum into plastic bags, dried at 55°C for 72 h in a forced-air oven, ground using a 0.2-mm sieve, and stored at -20°C. Urine output of AFM₁ was calculated as AFM₁ excretion in urine, µg/d = AFM₁ in urine, µg/L, × urine yield, kg/d, where urine yield, kg/d (Bannink et al., 1999) = $1.3441 + DMI, kg/d \times (1.079 \times Na, \% \text{ of diet DM} + 0.5380 \times K, \% \text{ of diet DM} + 0.1266 \times N, \% \text{ of diet DM}) - \text{milk yield, kg/d} \times (0.1216 + 0.0275 \times \text{milk protein, } \%)$, and dietary nitrogen was calculated using the Kjeldahl method as diet N, % = diet CP, % ÷ 6.25. Free fecal AF, µg/d = free AF in feces, µg/kg × feces yield, kg/d, where feces yield, kg/d = DMI, kg/d × diet TDN, %.

Laboratory Analysis of AFM₁. Milk samples were thawed, then centrifuged at $1,875 \times g$ at 4°C for 15 min, and the fat layer was removed. Ten milliliters of skim milk samples were passed through AFLAPREP M immunoaffinity cleanup columns (R-Biopharm Rhone Ltd., Glasgow, Scotland), columns were washed with 10 mL of PBS, and AFM₁ was then eluted from the columns with 1.5 mL of acetonitrile, followed by 1.5 mL of water. Eluates were combined, placed in autosampler vials, and analyzed by HPLC with fluorescence detection. The HPLC system (Hitachi High Technologies America Inc., Schaumburg, IL) consisted of a Hitachi model L-7100 pump with a Hitachi Model L-7485 fluorescence detector (365 nm excitation and 440 nm emission), a Hitachi Model L-7200 autosampler with Hitachi D-7000 data acquisition interface, and Concert Chrom software on a microcomputer. The HPLC column was a 150×4.6 mm reversed-phase HyperClone 3-µm C₁₈ BDS column (Phenomenex, Torrance, CA) with a C₁₈ SecurityGuard precolumn (Phenomenex). The mobile phase was acetonitrile:methanol:water (15:15:70) run at 1 mL/min. The injection volume was 50 µL for all standards and samples. The detection limit was set at 40 ng/kg of AFM₁. Urine samples were thawed, centrifuged at $1,875 \times g$ at 4°C for 15 min, and 2 mL of the

urine was analyzed for AFM₁ using the same procedure described above.

Laboratory Analysis of Fecal Aflatoxins. Fecal samples were thawed and quantified for total AF (B₁ + B₂ + G₁ + G₂) using HPLC. Five grams of each fecal sample was extracted with 80% aqueous methanol for 30 min and 2 mL of supernatant was combined with 10 mL of PBS buffer and passed through EASI-EXTRACT aflatoxin immunoaffinity columns (R-Biopharm Inc., Washington, MO). Columns were washed with 10 mL of PBS, and AF was then eluted from the columns with 1.5 mL of methanol, followed by 1.5 mL of water. Eluates were combined, placed in autosampler vials, and analyzed by HPLC with fluorescence detection. The HPLC system was an Elite LaChrom (Hitachi High Technologies America Inc.) with model L-2485 fluorescence detector, model L-2000 autosampler, and Hyperclone 3µm 100 × 4.6 mm column, fitted with a KobraCell (R-Biopharm Rhône Ltd., Glasgow, UK) at 50µA. The mobile phase consisted of 2,300 mL of distilled water, 500 mL of methanol, 500 mL of acetonitrile, 1.2g of potassium bromide, and 360µL of nitric acid. The flow rate was stabilized at 0.9 mL/min and the detector was set for excitation of 360 nm and emission of 440 nm. A total AF standard (B₁, B₂, G₁, and G₂, in a ratio of 4/1/4/1, Trilogy Analytical Laboratory, Washington, MO), 5µg/mL in acetonitrile, was used to establish peak retention times. The detection limit for all AF was 5 ng/mL.

Statistical Analysis

Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC). Variables were first tested for normality using the UNIVARIATE procedure. Somatic cell count data were log₁₀-transformed, but all other variables were normally distributed. Next, data were analyzed as a randomized complete block design using the MIXED procedure with repeated statement. The statistical model included fixed effects of treatment, time, and their interaction. Treatment within block was the random effect. Time was the specific term for the repeated statement, with cow within treatment and block as subject. Either autoregressive order 1 or compound symmetry were the covariance structures selected based on the lowest Akaike's information criterion (Littell et al., 1998). Averaged data from d -1 and 0 of the trial (adaptation period) were used as covariates for each variable. Three orthogonal contrasts were used to interpret treatment effects: (1) absence versus presence of AF in the diet (NC vs. PC, TN, and UP), (2) presence of AF alone versus presence of AF and additives (PC vs. TN and UP), and (3) Toxy-Nil versus Unike Plus (TN vs. UP). The PDIFF option was

used to separate least squares means. Significance was declared at $P \leq 0.05$ and a tendency was noted when $0.05 < P \leq 0.10$. Results are reported as least squares means $\pm$ standard error of the mean.

RESULTS AND DISCUSSION

Average intakes of AF and additives are shown in Table 2. The dosage of AF (2.8 mg/cow per d) allocated to cows on PC, TN, and UP treatments was intended to provide a dietary concentration of 100 $\mu\text{g/kg}$ of diet DM, based on expected DMI of 28 kg/d. Actual DMI did not differ among treatments but was slightly lower than projected. This resulted in calculated dietary concentrations of AF intake that were similar among the 3 treatments that included AF, all of which were higher than NC. Dietary concentrations of AF averaged 106.5, 107.6, and 102.5 ± 2.9 $\mu\text{g/kg}$ of DM for PC, TN, and UP, respectively (Table 2). Concentrations of AF consumed differed slightly over time and there was an interaction of treatment and time, both due to daily variation in DMI. Intakes of TN and UP were similar, averaging 0.4 and $0.4 \pm 0.1\%$ of diet DM, respectively ($P = 0.09$). Additive intake also varied over time and there was an interaction of treatment and time (Table 2). Like AF intake, these differences reflected variation in daily DMI (Table 3). Examination of daily AF, TN, and UP intakes confirmed that they varied together over time (data not shown).

Dietary AF that is absorbed from the gut is metabolized in the liver to form AFM₁, which is the form secreted into milk or voided in urine (Kuilman et al., 2000). As expected, cows fed AF secreted substantial quantities of AFM₁ into their milk. Concentrations of AFM₁ in milk were consistent with those reported by Kutz et al. (2009), who also fed AF at about 100 $\mu\text{g/kg}$ of DM, but were markedly higher than those reported by others (Queiroz et al., 2012; Ogunade et al., 2016), who fed about 75 μg of AF/kg of DM, indicating a large difference in absorption, probably due in part to concentrations of AF fed. Inclusion of either TN or UP in the diet significantly reduced ($P < 0.01$) the concentration and mass of AFM₁ secreted in milk by 50 to 60%, relative to cows on PC treatment, and no statistical difference was observed between TN and UP. Both of these variables were significantly affected by time and time by treatment interactions (Table 2). As shown in Figure 1, concentration and mass of AFM₁ in milk decreased rapidly after withdrawal of AF from diets, as reported previously (Masoero et al., 2007; Queiroz et al., 2012; Ogunade et al., 2016). For TN and UP, concentrations dropped below the FDA limit of 0.5 $\mu\text{g/kg}$ of milk within 24 h (d 8) and approached 0 $\mu\text{g/kg}$ of milk by 48 h (d 9), whereas concentration and mass in

PC appeared to decline more rapidly but still required 1 d more than TN and UP to drop below the FDA limit (d 9) or approach zero (d 10). It is clear from Figure 1 that the main difference between treatments with (TN and UP) or without (PC) additives is the concentration or mass of AFM₁ in milk on d 7 and 8, presumably reflecting lower absorption of AF from the gut in TN and UP due to binding effects of these additives. This is supported by the much lower transfer of AF from feed to milk in the presence of these additives, which did not vary over time (Table 2). Furthermore, expressing clearance of AFM₁ via milk as a daily percentage of the average content in milk on d 6 and 7 revealed no difference between PC, TN, and UP, as shown clearly in Figure 2. These results suggest that TN and UP did not alter metabolism of absorbed AF to AFM₁, nor its transfer into, and secretion from, the mammary glands but rather reduced uptake from the gut. This is in agreement with the similar clearance of AFM₁ in the presence or absence of a mycotoxin-sequestering agent found by Queiroz et al. (2012) but is in contrast to the study of Ogunade et al. (2016), who reported that 3 mycotoxin-sequestering agents evaluated did not reduce AFM₁ concentrations in milk during the dosing period but hastened their decline postdosing.

In the present study, concentrations of AFM₁ in urine were much higher than those in milk, and were also reduced ($P < 0.03$) by TN and UP relative to PC. Moreover, they did not differ between TN and UP (Table 2). On the other hand, the reductions in concentration of AFM₁ in urine of TN or UP, relative to PC, did not closely match the corresponding reductions in milk AFM₁, perhaps because urine was only collected at a single time point. Few others have investigated urinary excretion of AFM₁, but Sulzberger et al. (2017) also found higher concentrations of AFM₁ in urine than milk and reported that, whereas dietary additives reduced AFM₁ in milk, there was no such effect on urinary concentrations. Similarly, Weatherly et al. (2018) found higher concentrations of AFM₁ in urine compared with milk, although the dietary additives tested in that study were not effective in reducing concentrations of AFM₁ in milk or urine. It is clear that AFM₁ is excreted via urine to a greater extent than via milk, but factors regulating the relative uptake by kidney and mammary gland, and how they may be affected by dietary additives such as mycotoxins-sequestering agents, are unknown. We also found that the sequestrants reduced concentrations of AF in feces, relative to PC (Table 2). This result is counterintuitive to the assumption that the mycotoxin-sequestering additives reduced uptake by sequestering AF in the gut but is probably explained by additive-bound AF being undetectable by our analysis as a result of the tight binding

Table 2. Effects of aflatoxin (AF) and dietary mycotoxin-sequestering additives on AF intake, excretion, and clearance in lactating dairy cows

Variable	Treatment ¹				P-value				Contrast ³		
	NC	PC	TN	UP	SEM ²	Treatment	Time	Interaction	1	2	3
Intake of AF, µg/kg of diet	0.0 ^b	106.5 ^a	107.6 ^a	102.5 ^a	2.9	<0.01	<0.01	0.01	<0.01	0.68	0.22
Intake of sequestrant, % of diet	0.0 ^a	0.0 ^a	0.4 ^b	0.4 ^b	0.0	<0.01	<0.01	<0.01	<0.01	<0.01	0.09
Milk ⁴											
AFM ₁ concentration, µg/kg	0.0 ^c	0.6 ^b	0.2 ^a	0.3 ^a	0.1	<0.01	<0.01	<0.01	<0.01	<0.01	0.43
AFM ₁ excretion, µg/d	0.0 ^c	20.5 ^b	8.1 ^a	9.8 ^a	1.7	<0.01	<0.01	<0.01	<0.01	<0.01	0.48
AF transfer, %	0.0 ^c	2.7 ^a	1.0 ^b	1.3 ^b	0.2	<0.01	0.12	0.77	<0.01	<0.01	0.40
Clearance of AFM ₁ , %/d	0.0 ^b	46.1 ^a	66.5 ^a	50.0 ^a	6.7	<0.01	<0.01	<0.01	<0.01	0.15	0.09
Urine ⁷											
AFM ₁ concentration, µg/L	0.5 ^c	14.2 ^a	6.9 ^b	7.4 ^b	1.5	<0.01	—	—	<0.01	<0.01	0.83
AFM ₁ excretion, µg/d	15.4 ^c	521.6 ^a	225.8 ^b	250.8 ^b	53.1	<0.01	—	—	<0.01	<0.01	0.75
AF transfer, %	0.0 ^c	18.6 ^a	8.0 ^b	9.0 ^b	1.9	<0.01	—	—	<0.01	<0.01	0.75
Feces ⁷											
Free AF concentration, µg/kg	0.2 ^c	12.4 ^a	7.7 ^b	8.9 ^b	0.6	<0.01	—	—	<0.01	<0.01	0.21
Free AF excretion, µg/d	1.1 ^c	95.6 ^a	57.8 ^b	69.6 ^b	4.8	<0.01	—	—	<0.01	<0.01	0.09
AF transfer, %	0.0 ^c	3.4 ^a	2.1 ^b	2.5 ^b	0.2	<0.01	—	—	<0.01	<0.01	0.09

^{a-c}Means within a row with different superscript letters differ ($P \leq 0.02$).¹Treatments fed per cow per day: NC = 0 mg of AF + 0 g of sequestrant; PC = 2.8 mg of AF + 0 g of sequestrant; TN = 2.8 mg of AF + 100 g of Toxy-Nil (Nutriad Animal Feed Additives, Dendermonde, Belgium); UP = 2.8 mg of AF + 100 g of Unike Plus (Nutriad).²Pooled SEM.³Orthogonal contrasts: contrast 1 = absence versus presence of AF (NC vs. PC, TN, and UP); contrast 2 = presence of AF alone versus AF plus sequestrants (PC vs. TN and UP); contrast 3 = comparison of sequestrant Toxy-Nil and Unike Plus (TN vs. UP).⁴Results represent the average of the last 2 d of the challenge period (d 6 and 7) plus the 7 d of the postchallenge period (d 8 to 14).⁵AF transfer, % = AFM₁ excretion in milk or urine, µg/d, or free AF excretion in feces, µg/d ÷ intake of AF, µg/d × 100.⁶Clearance of AFM₁ from milk, %/d = averaged AFM₁ excretion in milk until nondetected levels, µg/d ÷ averaged AFM₁ in milk on d 6 and 7, µg × 100.⁷Results represent the last day of the challenge period.⁸AFM₁ excretion in urine, µg/d = AFM₁ in urine, µg/L × urine yield, kg/d, where urine yield, kg/d (Bannink et al., 1999) = 1.3441 + DMI, kg/d × (1.079 × Na, % of diet DM + 0.5380 × K, % of diet DM + 0.1266 × N, % of diet DM) – milk yield, kg/d × (0.1216 + 0.0275 × milk protein, %).⁹AF in feces, µg/d = AF in feces, µg/kg × feces yield, kg/d, where feces yield, kg/d = DMI, kg/d × diet TDN, %.

Table 3. Effects of aflatoxin (AF) and dietary inclusion of mycotoxin-sequestering additives on performance and general health variables of lactating dairy cows

Variable	Treatment ¹				SEM ²	P-value			Contrast ³		
	NC	PC	TN	UP		Treatment	Time	Interaction	1	2	3
DMI, kg/d	26.2	26.4	27.2	27.8	0.8	0.41	<0.01	0.62	0.28	0.24	0.60
Milk measurement											
Milk, kg/d	34.5	36.3	35.5	35.0	0.7	0.38	<0.01	0.41	0.22	0.25	0.62
ECM, ⁴ kg/d	35.4	37.1	36.1	37.8	1.7	0.73	0.50	0.41	0.41	0.93	0.47
3.5% FCM, ⁵ kg/d	34.9	36.7	35.5	38.9	1.8	0.60	0.27	0.37	0.38	0.96	0.32
Fat, %	3.61	3.54	3.49	4.05	0.17	0.10	<0.01	0.18	0.68	0.27	0.03
Fat, kg/d	1.24	1.28	1.23	1.43	0.08	0.32	0.05	0.30	0.47	0.65	0.11
Protein, %	3.10	3.09	3.05	3.01	0.04	0.35	0.09	0.74	0.30	0.19	0.50
Protein, kg/d	1.08	1.11	1.07	1.06	0.04	0.85	0.18	0.71	0.92	0.40	0.82
Lactose, %	4.81	4.80	4.82	4.78	0.02	0.41	0.29	0.10	0.59	0.88	0.12
Lactose, kg/d	1.70	1.74	1.67	1.69	0.06	0.92	0.19	0.53	0.94	0.51	0.84
MUN, mg/dL	13.8	13.0	13.5	14.2	0.4	0.17	0.12	0.03	0.62	0.06	0.21
SCC, log ₁₀ (cells/mL)	4.46	4.42	4.54	4.30	0.14	0.65	0.03	0.19	0.80	0.98	0.23
Feed efficiency											
Milk/DMI	1.35	1.40	1.29	1.29	0.04	0.25	<0.01	0.64	0.74	0.05	0.99
ECM/DMI	1.37	1.42	1.35	1.41	0.07	0.84	0.01	0.61	0.70	0.61	0.53
3.5% FCM/DMI	1.36	1.42	1.32	1.42	0.07	0.67	0.03	0.74	0.74	0.54	0.31
Body measurement											
Milk flow rate, kg/min	2.9	3.0	2.9	3.0	0.1	0.93	<0.01	0.98	0.62	0.81	0.72
BW, kg	666	671	672	677	5	0.37	0.07	0.20	0.14	0.54	0.47
BCS, 1 to 5 points	3.1	3.2	3.3	3.2	0.06	0.17	0.05	0.80	0.03	0.58	0.88
Energy balance, ⁶ MJ/d	34.0	28.5	37.8	44.2	6.6	0.42	0.20	0.34	0.72	0.14	0.50
Lameness score, ⁷ 1 to 5 points	1.0	1.0	1.0	1.0	0.02	0.53	0.92	0.25	0.46	0.21	1.00
Rectal temperature, °C	38.6	38.5	38.6	38.5	0.1	0.79	<0.01	0.58	0.54	0.74	0.48

¹Treatments fed per cow per day: NC = 0 mg of AF + 0 g of sequestrant; PC = 2.8 mg of AF + 0 g of sequestrant; TN = 2.8 mg of AF + 100 g of Toxy-Nil (Nutriad Animal Feed Additives, Dendermonde, Belgium); UP = 2.8 mg of AF + 100 g of Unike Plus (Nutriad).

²Pooled SEM.

³Orthogonal contrasts: contrast 1 = absence versus presence of AF (NC vs. PC, TN, and UP); contrast 2 = presence of AF alone versus AF plus sequestrant (PC vs. TN and UP); contrast 3 = comparison of sequestrant Toxy-Nil and Unike Plus (TN vs. UP).

⁴ECM, kg/d = (milk yield, kg/d × 0.327) + (milk fat, kg/d × 12.86) + (milk protein, kg/d × 7.65).

⁵3.5% FCM, kg/d = (milk yield, kg/d × 0.4324) + (milk fat, kg/d × 16.2162).

⁶Calculated according to NRC (2001) as described in Materials and Methods.

⁷According to Sprecher et al. (1997), where 1 = normal gait and 5 = severely lame.

Figure 1. Time course of changes in concentrations of aflatoxin (AF) M₁ in milk of control cows (NC) or cows challenged with 2.8 mg/d of dietary AF alone (PC) or with dietary mycotoxin-sequestering additives Toxy-Nil (TN; Nutriad Animal Feed Additives, Dendermonde, Belgium) or Unike Plus (UP; Nutriad). Results shown are the last 2 d of AF challenge (d 6 and 7) and the 7 d postchallenge, shown as d 8 to 14. The dotted line at 0.5 µg/mL represents the FDA limit for AFM₁ residues in milk. Each data point represents the mean of 8 cows. Effects of treatment, time, and their interaction were significant ($P \leq 0.001$). Error bars represent SEM.

of AF to the additive, and the failure of the extraction method to release AF from the AF-additive complex. This conclusion is supported by the study of Phillips et al. (1988) who reported that once radiolabeled AF was sorbed to a mycotoxin-sequestering additive (aluminosilicate), it remained bound to the additive even after a series of eluotropic solvents were used to extract the AF. Sulzberger et al. (2017) reported that fecal concentrations of AF decreased with increasing dose of clays in the diet and suggested that clay-bound AF may be undetectable. Recently, Weatherly et al. (2018) also reported that a yeast and bentonite clay-based adsorbent tended to reduce AF in feces. Further studies on the effects of mycotoxin-sequestering additives on metabolism and clearance of AFM₁ via milk or urine appear warranted.

There is a marked difference in the efficacy of various mycotoxin-sequestering additives to reduce AF transfer from feed into milk. Based on studies in vitro, AF binding efficiency varies from 20 to 100%, depending on the product tested and on presence or absence of other compounds in incubation fluid (Diaz et al., 2002; Moschini et al., 2008; Gallo and Masoero, 2010). The same holds true in vivo, with results depending on the dosage and mode of AF delivery and on the specific additive and dosage tested. For example, Kutz et al. (2009) reported that of 3 mycotoxin-sequestering additives tested, 2 significantly reduced AFM₁ in milk, and 1 had no effect. Ogunade et al. (2016) tested 3 sequestering agents and found that none reduced AFM₁ in milk during AF challenge, but all hastened the decline in concentrations postchallenge. We found that both additives markedly reduced the percentage of AF transferred into milk, from 2.7% in PC to 1.0 and $1.3 \pm 0.2\%$ for TN and UP, reductions of 63 and 52%, respectively ($P < 0.001$). The reduction in percentage transfer is similar to that reported for other mycotoxin-sequestering additives (transfers of 0.32 to 3.68%, reductions of 27–70%; Kutz et al., 2009; Sulzberger et al., 2017; Xiong et al., 2018), but is comparatively high, indicating the efficacy of TN or UP in reducing AF residues in milk.

Concentrations of AF in the diet were about 5 times greater than the FDA limit for AF in feedstuffs and the concentration of AFM₁ in milk and urine was de-

Figure 2. Clearance of aflatoxin (AF) M₁ via secretion into milk of control cows (NC) or cows challenged with 2.8 mg/d of dietary AF alone (PC) or with dietary mycotoxin-sequestering additives Toxy-Nil (TN; Nutriad Animal Feed Additives, Dendermonde, Belgium) or Unike Plus (UP; Nutriad). Results represent the mass of AFM₁ secreted into milk on each day as a percentage of the average mass of AFM₁ in milk on the last 2 d of AF challenge (d 6 and 7). Results shown are the last 2 d of AF challenge (d 6 and 7) and the 7 d postchallenge, shown as d 8 to 14. Each data point represents the weighted mean of p.m. and a.m. milkings for 8 cows. The overall treatment effect was significant ($P < 0.001$) due to NC differing from PC, TN, and UP, but the latter 3 treatments did not differ. Error bars represent SEM.

pendent on feeding of AF and supplemental TN or UP, in effect creating an apparent dose relationship of AF uptake. In spite of this, there were no differences in DMI, milk yield, feed efficiency, or milk flow rate ($P \geq 0.25$; Table 3), with the exception of the comparison of PC to the mean of TN and UP for feed efficiency ($P = 0.05$). Our results were consistent with others who found no effect on DMI and milk yield when cows received dietary AF at 75 to 112 $\mu\text{g}/\text{kg}$ (Kutz et al., 2009; Queiroz et al., 2012; Xiong et al., 2018), although effects of mycotoxin binders on DMI seem to be dose dependent (Sulzberger et al., 2017). In contrast, reduction of DMI was observed at a concentration of 170 $\mu\text{g}/\text{kg}$ of AFB₁ in diet DM (Stroud, 2006). Others reported that AF challenge tended to decrease 3.5% FCM and ECM (Sulzberger et al., 2017), and tended to reduce indices of feed efficiency because of differences in milk composition (Ogunade et al., 2016; Sulzberger et al., 2017). Our results showed no effect of AF alone or with TN and UP on milk composition (Table 3), with 2 exceptions; there were tendencies ($P = 0.10$) for a treatment effect on milk fat percentage, which was due to a difference ($P = 0.03$) between groups TN and UP, and for an interactive effect of treatment and time on lactose content. We have no explanation for these findings, but some have reported indications of effects on fat percent (Maki et al., 2017; Sulzberger et al., 2017), whereas other studies did not find a difference in milk fat content in cows consuming AF and mycotoxin-sequestering additives (Queiroz et al., 2012; Xiong et al., 2015; Weatherly et al., 2018). Sulzberger et al. (2017) reported a negative linear effect of supplemental clay dosage on milk lactose contents and yield, relative to AF alone. However, that study also found a similar negative effect on milk yield. It would appear that such results reflect subtle and unexplained effects of AF and sorbents or experimental variation. We also found an interactive effect of treatment and time on MUN ($P = 0.03$; Table 3), apparently due to a difference between PC versus TN and UP ($P = 0.06$). Concentrations of MUN are typically related to levels of dietary CP (Reid et al., 2015) and reflect protein metabolism but 3 other studies on AF and mycotoxin binders did not find any effect on MUN (Maki et al., 2016; Sulzberger et al., 2017; Weatherly et al., 2018).

Previous studies have variously reported effects of AF and mycotoxin-sequestering additives on DMI, milk yield, markers of immune function, or metabolism (Queiroz et al., 2012; Maki et al., 2016; Ogunade et al., 2016), and recently, on cow behavior (Sulzberger et al., 2017). Thus, we evaluated several variables related to general cow health and performance. Treatments had no effects on BW, EB, lameness score, rectal temperature, or milk flow rate (Table 3). The relatively short

duration of our experiment made it unlikely to detect changes in variables such as BW, BCS, and lameness. Nevertheless, DMI ($P < 0.01$), BCS ($P = 0.05$), BW ($P = 0.07$), and rectal temperature ($P < 0.001$) were all affected by time. In addition, although there was no overall treatment effect, the orthogonal contrast comparing the absence of dietary AF to the 3 treatments that included AF (NC vs. PC, TN, and UP) was significant for BCS ($P = 0.03$). Most other studies on effects of dietary AF have not examined BW or BCS but 2 recent reports found no effects (Maki et al., 2016; Sulzberger et al., 2017). In our experiment, cows were in mid-lactation and both milk yield and DMI declined over time (data not shown), thus it is not clear why cows increased in BCS and tended to gain BW, but those effects were small and probably of no biological significance.

CONCLUSIONS

We conclude that inclusion of 100 g/cow per d of TN or UP in the diet significantly reduced (50 to 60%) AFM₁ concentrations in milk of cows consuming TMR containing approximately 5 times the FDA limit of 20 μg of AF/kg of diet DM. Supplementation with TN or UP also reduced the time required postchallenge for AFM₁ concentrations to drop below the FDA limit for milk. Results suggest that both TN and UP reduced absorption of AF from the gastrointestinal tract.

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