PEER REVIEWED

Dose response reduction of aflatoxin M_1 in milk of Holstein cows administered an aluminosilicate clay adsorbent

S. C. Allen, MS[1]; **K. Russo,** DVM[2]; **D. M. Paulus Compart,** PhD[2]; **D. Diaz,** PhD[3]; **S. H. Ward,** PhD[1]
[1] Department of Animal Science, North Carolina State University, NC 27695
[2] PMI, Arden Hills, MN 55126
[3] School of Animal and Comparative Biomedical Science, University of Arizona, Tucson, AZ 85721
Corresponding author: Dr. Stephanie H. Ward, Department of Animal Science, 111D Polk Hall, Box 7621, NC State University, Raleigh, NC 27695; shward@ncsu.edu; Phone: 919-515-4015

Abstract

Thirty-five Holstein cows were utilized in a completely randomized design to evaluate the efficacy of 2 doses of an aluminosilicate clay at reducing aflatoxin M_1 (AFM_1) transfer into milk. Cows were stratified by parity, stage of lactation, and milk production. Cows were assigned to 1 of 5 dietary treatments for 13 days (n = 7): (1) control (CON), basal diet; (2) clay control (4C), CON plus 4 oz clay; (3) aflatoxin (AF) control (AF-CON), CON plus 113 ppb AF; (4) AF-CON diet with 4 oz clay (4C+AF); or (5) AF-CON diet with 8 oz clay (8C+AF). Data were analyzed using the GLM procedure of SAS, and significance was declared when $P \leq 0.05$. Milk yield was greatest in 4C+AF and 8C+AF cows and least in CON. Milk AFM_1 concentration averaged < 0.01, N/D (< 0.04 ppb), 1.64, 1.26, and 0.90 ppb for CON, 4C, AF-CON, 4C+AF, and 8C+AF diets, respectively. A dose response was observed for AFM_1 transfer with a 21.88 and 40.63% reduction in cows consuming 4C+AF and 8C+AF diets, respectively. Feeding aluminosilicate clay to AF challenged Holstein cows resulted in a dose response reduction in AFM_1 secretion and improved milk production.

Key words: aflatoxin, aluminosilicate clay, dairy cow, milk production, mycotoxins

Résumé

On a utilisé 35 vaches Holstein dans un plan aléatoire complet afin d'évaluer l'efficacité de deux doses d'argile aluminosilicate pour réduire le transfert d'aflatoxine M_1 (AFM_1) dans le lait. Les vaches ont été stratifiées par la parité, le stade de lactation et la production de lait. Les vaches ont été attribuées à l'un des 5 traitements pendant 13 jours (n = 7) : (1) témoin (**CON**), diète de base; (2) témoin d'argile (**4C**), CON plus 4 onces d'argile; (3) témoin d'aflatoxine (AF) (**AF-CON**), CON plus 113 ppb AF; (4) diète AF-CON avec 4 onces d'argile (**4C+AF**); ou (5) diète AF-CON avec 8 onces d'argile (**8C+AF**). Les données ont été analysées avec la procédure GLM de SAS et la valeur du seuil alpha était de 0.05. La production de lait était la plus élevée chez les vaches des groupes 4C+AF et 8C+AF et la moins élevée chez les vaches du groupe CON. La concentration d'AFM_1 dans le lait était en moyenne < 0.01, non-détectable (< 0.04 ppb), 1.64, 1.26 et 0.90 ppb dans les groupes CON, 4C, AF-CON, 4C+AF, and 8C+AF, respectivement. Il y avait une effet dose-réponse pour le transfert d'AFM_1 car on observait une réduction de l'ordre de 21.88% chez les vaches du groupe 4C+AF et une réduction de 40.63% chez les vaches du groupe 8C+AF. L'utilisation d'argile aluminosilicate chez des vaches Holstein ingérant de l'AF a entrainé une réduction dose-réponse de la sécrétion d'AFM_1 et augmenté la production de lait.

Introduction

Aflatoxins (AF) commonly found in dairy feeds are secondary metabolites primarily produced by species within the Aspergillus genus. Aflatoxin occurs naturally in 4 forms: aflatoxin B_1 (AFB_1), B_2, G_1, and G_2. Aflatoxin B_1 is a potent naturally occurring carcinogen, and if consumed by lactating animals, can be transferred into the milk in the form of aflatoxin M_1 (AFM_1). When consumed by humans, aflatoxin B_1 is classified as a group 1 carcinogen, and AFM_1 is classified as a group 2B carcinogen.[10] Processing of milk has variable results on AFM_1 concentration,[16,20] and AFM_1 has been observed in numerous food products including infant formula, dried milk, cheese, yogurt, and milk products from various animals, including human breast milk.[8] In the United States, action limits of 0.5 ppb AFM_1 and 20 ppb aflatoxin B_1 in milk and lactating dairy cow feeds, respectively, have been established.[6] Over 100 nations have set regulatory limits on allowable aflatoxin levels in human food or animal feed.[23]

Contamination of dairy diets can be a large problem for dairy herds, and several methods of preventing AFM_1 in milk have been investigated. Some pre-harvest methods

proposed include the use of genetically engineered crops[9] or compounds that may inhibit the growth of AF producing molds.[19] Post-harvest methods, like the mitigation through aluminosilicate clay adsorbents, have been more successful in commercial operations. Aluminosilicate clays can reduce the toxic effects of AFB_1[18] by binding to the toxin prior to absorption by the small intestine. For a sequestering agent to be effective, it must tightly bind with AF in the feed and/or foregut without the bond being damaged or dissociating in the harsh conditions of the digestive tract within the animal. If successful, the sequestering agent complex will pass through the digestive tract and be excreted in the feces, preventing or minimizing the animal's exposure time to the carcinogen.[3] Dietary addition of sequestering agents has been reported to reduce the transfer of AFM_1 into the milk of cows consuming diets containing AFB_1 without negatively affecting production.[4,5,11,12,13,17]

The objective of the current study was to evaluate the impact of 2 concentrations of a hydrated sodium calcium aluminosilicate clay[a] in lactating dairy cow diets contaminated with AFB_1 on the presence of AFM_1 in milk and on the cows' production parameters. Additionally, the study aimed to determine the effects of the aluminosilicate clay on body condition, weight, and respiratory rates of cows consuming AFB_1 contaminated feed.

Materials and Methods

Experimental Design

This study was conducted at the North Carolina Department of Agriculture and Consumer Services Piedmont Research Station (Salisbury, NC) in February 2018. Thirty-five lactating Holstein cows were utilized in a randomized complete block design. A power analysis was conducted using the POWER procedure of SAS (Cary, NC) using data previously generated by these authors. Seven cows per treatment were considered sufficient to determine significance; however, consideration also had to be given to number of individual feed bins available at the facility. Cows were stratified by parity, stage of lactation, and previous milk production. Cows averaged 67.35 lb (30.55 kg) milk yield and 189 days-in-milk (DIM) at the start of the study. This protocol was approved by the NC State University Animal Care and Use Committee (IACUC#, 17-169A).

Cows were predicted to consume 66 lb (30 kg) of dry matter intake (DMI), based on average DMI during the week prior to study start, and 0.0066 lb (3 g) AF was added to diets to achieve 100 ppb AF; however, intake was less than predicted (58.58 lb/d, 26.57 kg/d), resulting in an average AF concentration of 113 ppb. Cows were randomly assigned (round robin style, by picking cow numbers at random) to 1 of 5 dietary treatments (n = 7): (1) control (CON), basal total mixed ration (TMR; Table 1) with no AF or aluminosilicate clay; (2) clay control (4C), basal TMR plus 4 oz aluminosilicate clay; (3) AF control (AF-CON), basal TMR plus 113 ppb AF; (4) AF diet with low aluminosilicate clay dose (4C+AF), basal TMR plus 4 oz clay and 113 ppb AF; or (5) AF diet with high clay dose (8C+AF) basal TMR plus 8 oz clay and 113 ppb AF. Aflatoxin B_1 (University of Missouri, Columbia, MO) produced using rice fermentation by *A. parasiticus* NLRR 2999 according to the methods described by Shotwell et al[20] was utilized in this study.

Table 1. Dietary composition of basal diet.

Feedstuff	Inclusion, % DM
Corn silage	42.97
Corn grain	19.97
Soybean meal, 48 %	17.47
Whole cottonseed	13.61
Cottonseed hulls	3.36
Custom mineral mix*	2.59

* Contains calcitic limestone, mono-dicalcium phosphate, Dynamate (The Mosaic Company, Plymouth, MN), sodium bicarbonate, vitamin trace mineral supplement, and salt

Cows were individually fed once daily at 1000 hour in individual feeding gates,[b] allowing for ad libitum intake. All treatment additions were top dressed and mixed into approximately the top third of feed offered for 13 days. Cows were milked at 0800 and 2000 hours in a double seven herringbone milking parlor, and milk from cows on this study was discarded throughout the study and for 3 milkings after the last day of treatment diets were administered.

Body Weight, Body Condition, Locomotion, and Respiratory Rates

Cows were weighed daily following milking.[c] Body condition score (BCS), locomotion score, and respiratory rates were evaluated by the same observer on days 3, 5, 9, and 13 of the study after milking at 1200. Body condition was measured in 0.25 unit increments on a 1 to 5 scale.[7] Locomotion was measured in 1.0 unit increments on a 1 to 5 scale.[26] Body condition and locomotion were similar across treatments at the start of the trial and averaged 2.89 and 1.23, respectively. Respiratory rate was determined by observing the number of breaths taken for 15 seconds and multiplied by 4 to produce respirations per minute.

Feed Analysis

Basal TMR from the start of the experiment was analyzed for aflatoxin B_1, B_2, G_1, G_2, vomitoxin, 3-Acetyl DON, 15-Acetyl DON, T-2 toxin, and zearalenone.[d] Feed and orts were sampled on days 4, 6, 10, and 12 and composited by week and treatment (days 4 and 6 vs d 10 and 12). Feed samples were placed in an oven at 149° F (65° C) until dry to determine air dry matter, which is used to calculate dry matter intake (DM). Samples were then ground through a 2 mm screen in a Thomas Wiley mill[e] and stored at room temperature. All feed samples were subjected to proximate analysis for total dry matter (DM; method 934.01[1]), ash

(method 942.05[1]), neutral detergent fiber (NDF; method 973.18[1]), acid detergent fiber (ADF; method 2002.04[1]), and crude protein[d] (CP). Organic matter was determined by subtracting ash from total DM.

Milk Analysis

Milk samples were collected at each milking on days 3, 5, 7, 9, 11, and 13 and composited by day. Samples were frozen at -4° F (-20° C) immediately after collection. Additionally, a second sample was collected on days 5 and 13 of the treatment period. Broad Spectrum Microtabs II™ tablets[f] were added to the additional samples after each milking for preservation of the sample. Additional samples from d 5 and 13 of the treatment period were analyzed for fat, protein, solids (SNF), and somatic cell counts (SCC).[g,1] Bently FTS Combi[h] was used to analyze SCC. Milk component yields were calculated daily by multiplying the concentration of milk components by milk yield. Feed efficiency (FE) was calculated using the following equation:

$$FE = lb\ milk/lb\ DMI$$

Somatic cell count was converted to somatic cell score (SCS) using the following equation:

$$SCS = LOG\ 2\left(\frac{SCC}{100}\right) + 3$$

Energy corrected milk (ECM) yield was calculated using the following equation:

$$ECM = (0.327 * milk\ lb) + (12.95 * fat\ lb) + (7.65 * protein\ lb)$$

Aflatoxin Analysis

Frozen milk samples were analyzed for AFM_1 concentration using HPLC with fluorescence detection[5] (University of Missouri, Columbia, MO). The detection limit was set at 0.04 ppb. Aflatoxin secretion was calculated by multiplying the milk AFM_1 concentration by total milk yield on the day of collection. Aflatoxin transfer was calculated for AF-CON, 4C+AF, and 8C+AF diets by dividing AFM_1 secretion by AF administered in feed and multiplying by 100. Calculations are shown by the following equations:

$$AF\ secretion = concentration\ of\ AFM1\ in\ milk \times milk\ yield$$

$$AF\ transfer = \left(\frac{\mu g\ AFM1\ secreted}{\mu g\ AFB1\ administered}\right) \times 100$$

Statistical Analysis

Data were analyzed using the GLM procedure of SAS.[i] Treatment and day were considered independent variables, and milk yield, FE, ECM, DMI, nutrient intakes, AFM_1 variables, milk composition, body weight, change in body weight, BCS, locomotion score, and respiratory rate were dependent variables. Previous milk yield and DHIA records from a test day 3 days prior to the start of the treatment period and were used as a covariate to adjust milk yield, fat, protein, solids, and somatic cell count. Means were separated using Fisher's Least Significant Difference, and significance was declared when $P \leq 0.05$. Tendencies were discussed when $P > 0.05$ and ≤ 0.10.

Results and Discussion

Feed Analysis and Intake

The basal diet was under the analytical detection limits for mycotoxin contamination (5 ppb) at the start of the study. Diets were similar across treatments in DM, NDF, ADF, and CP (Table 2). Ash was greatest in 8C+AF and least in AF-CON and 4C+AF diets, and all other diets were intermediate. Intake of dietary components is shown in Table 3. No treatment by day effects were observed in this study; however, there was an effect of day on DMI, but no pattern was observed (data not shown). Intake of all other nutrients was unaffected by day. Dry matter intake averaged 57.87 (26.25), 59.51(26.99), 57.08 (25.89), 58.23 (26.41), and 60.84 (27.60) ± 0.90 (0.41) lb (kg)/d for CON, 4C, AF-CON, 4C+AF, and 8C+AF cows, respectively ($P = 0.034$). Dry matter intake was greater for cows fed 8C+AF treatment relative to cows on the CON, AF-CON, and 4C+AF treatment, and cows fed 4C were intermediary. It is important to note, however, that DMI based on %BW

Table 2. Analyzed chemical composition of dietary treatments with differing levels of aflatoxin and aluminosilicate clay.

	Treatment*						
Item†	CON	4C	AF-CON	4C+AF	8C+AF	SEM‡	*P*-value
DM, %	52.46	52.56	51.97	51.98	52.45	2.00	0.681
CP, % DM	16.40	16.55	16.80	15.95	16.85	0.65	0.539
NDF, % DM	38.91	37.51	40.14	40.18	40.38	1.68	0.464
ADF, % DM	16.57	17.00	18.28	17.80	17.38	0.67	0.232
Ash, % DM	4.91[bc]	5.71[ab]	4.37[c]	4.55[c]	6.09[a]	0.40	0.019

* CON = basal TMR with no aflatoxin (AF) or aluminosilicate clay; 4C = basal TMR plus 4 oz aluminosilicate clay; AF-CON = basal TMR plus 113 ppb AF; 4C+AF = basal TMR plus 4 oz aluminosilicate clay and 113 ppb AF; 8C+AF = basal TMR plus 8 oz aluminosilicate clay and 113 ppb AF

† DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber

‡ Largest standard error of the mean

[a–b]Means within a row with different superscripts differ ($P < 0.05$).

Table 3. Intake of dietary components by dietary treatment with differing levels of aflatoxin and aluminosilicate clay.

	Treatment*						
Item,†	**CON**	**4C**	**AF-CON**	**4C+AF**	**8C+AF**	**SEM‡**	***P*-value**
DMI, lb	57.87[b]	59.51[ab]	57.08[b]	58.23[b]	60.84[a]	0.90	0.034
DMI, % BW	3.84	3.77	3.78	3.73	3.89	0.66	0.532
CP intake, lb	9.43[bc]	9.84[b]	9.04[c]	9.27[c]	10.27[a]	0.16	< 0.001
NDF intake, lb	22.39[c]	22.16[c]	22.71[bc]	23.33[b]	24.57[a]	0.36	< 0.001
ADF intake, lb	9.40[b]	10.03[a]	10.32[a]	10.27[a]	10.47[a]	0.16	< 0.001
Organic matter intake, lb	55.02	56.06	54.58	55.14	56.47	0.91	0.262

* CON = basal TMR with no aflatoxin (AF) or aluminosilicate clay; 4C = basal TMR plus 4 oz aluminosilicate clay; AF-CON = basal TMR plus 113 ppb AF; 4C+AF = basal TMR plus 4 oz aluminosilicate clay and 113 ppb AF; 8C+AF = basal TMR plus 8 oz aluminosilicate clay and 113 ppb AF

† DMI = dry matter intake; BW = body weight; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber

‡ Largest standard error of the mean

[a–b]Means within a row with different superscripts differ ($P < 0.05$).

was not different ($P = 0.532$) across treatments, indicating the potential that DMI differences could be related to body size and capacity and less to treatment variation. Increased DMI in cows consuming 8C+AF resulted in increased nutrient intakes (CP and NDF) compared to other treatments. Previous work reported no differences in nutrient intake following the addition of AF or clay in the diet.[9]

Milk Yield and Composition

Milk yield, milk composition, and feed efficiency by treatment are shown in Table 4. Cows consuming 4C and 8C+AF diets produced the most milk, and cows consuming CON diets produced the least milk. There was no interaction between treatment and day or impact of day on milk yield (data not shown). Previous studies evaluating sequestering agents in AF contaminated diets reported no changes in milk yield during treatment periods.[13,17,24] Pate et al[15] reported linear and quadratic response to milk yield with increasing clay dosage in cows administered 100 ppb AFB_1 for 3 days. Ogunade et al[14] reported a tendency for reduced milk production in cows challenged with AF and no clay compared to control cows (no AF or clay), and a decrease in milk yield in AF challenged cows supplemented with sodium bentonite clay compared to control cows. Similarly, Sulzberger et al[22] reported a decrease in milk yield as clay concentrations increased; however, there was no difference between control cows and cows administered AF with no clay. Xiong et al[25] exposed lactating cows to 20 ppb AFB_1 for 7 weeks and re-

Table 4. Milk yield, milk composition, and feed efficiency by dietary treatment with differing levels of aflatoxin and aluminosilicate clay.

	Treatment*						
Item†	**CON**	**4C**	**AF-CON**	**4C+AF**	**8C+AF**	**SEM‡**	***P*-value**
Milk yield, lb	62.52[b]	70.21[a]	66.03[ab]	65.99[ab]	69.00[a]	1.76	0.011
ECM, lb	68.56[b]	75.89[a]	79.08[a]	75.18[a]	76.22[a]	1.97	0.003
FE	1.08	1.20	1.17	1.14	1.18	0.03	0.071
Fat, lb	2.49[c]	2.76[b]	3.09[a]	2.90[ab]	2.85[ab]	0.09	<0.001
Fat, %	3.87	3.97	4.52	4.38	4.37	0.21	0.118
Protein, lb	2.06[bc]	2.27[a]	2.21[ab]	2.11[bc]	2.23[ab]	0.05	0.014
Protein, %	3.28	3.33	3.26	3.18	3.37	0.05	0.116
SNF, lb	5.62[c]	6.18[a]	5.90[abc]	5.78[bc]	6.11[ab]	0.14	0.037
SNF, %	8.92	8.90	8.82	8.76	8.94	0.06	0.195
Lactose, lb	3.00	3.29	3.16	3.10	3.26	0.08	0.093
Lactose, %	4.77	4.71	4.70	4.73	4.72	0.04	0.723
SCS	2.86	3.96	3.04	3.21	3.52	0.20	0.147

* CON = basal TMR with no aflatoxin (AF) or aluminosilicate clay; 4C = basal TMR plus 4 oz aluminosilicate clay; AF-CON = basal TMR plus 113 ppb AF; 4C+AF = basal TMR plus 4 oz aluminosilicate clay and 113 ppb AF; 8C+AF = basal TMR plus 8 oz aluminosilicate clay and 113 ppb AF

† ECM = energy corrected milk ([0.327 * milk lb] + [12.95 * fat lb] + [7.65 * protein lb]); FE = feed efficiency (kg dry matter intake / kg milk); SNF = solids not fat; component yields calculated daily by multiplying milk yield by component percent; SCS = somatic cell score (LOG 2 (somatic cell count x 10^3/100) + 3)

‡ Largest standard error of the mean

[a–b]Means within a row with different superscripts differ ($P < 0.05$).

ported no effect of AF intake on milk yield. However, those studies, as well as the current study, utilized cultured AFB_1. Applebaum et al[2] reported a decrease in milk yield averaging 5.3 lb (2.4 kg) in cows fed naturally contaminated AFB_1 (a crude protein extract that contained AFB_1 with other aflatoxins and metabolites) for 7 days. The difference in production responses to mycotoxicosis is most likely attributed to the interaction between multiple mycotoxins that may occur in naturally contaminated feed compared to cultured AFB_1 that is often used in research.

Cows consuming CON diet produced the least ECM compared to all other diets. There was a tendency for reduced FE of CON cows compared to AF-CON, 4C, and 8C+AF cows. No interaction between treatment and day or impact of day was observed for FE.

Milk fat percent, protein percent, lactose percent, SNF percent, and SCC were similar across treatments. This is consistent with previous research.[5,11,12,13] However, Queiroz et al[17] reported a decrease in milk protein (%) in cows consuming AF with no clay. In addition, Queiroz et al[17] also reported cows consuming a high dose of clay (1% DMI) produced more milk protein relative to cows consuming a low dose of clay (0.2%), although both were similar to control diets. No interaction between treatment and day or impact of day was observed for milk components with the exception of fat percent. Fat percent was greater in samples taken on day 13 compared to day 5 (4.31 vs 4.01 ± 0.13; $P = 0.022$).

Protein, fat, lactose, and SNF yield were affected by treatment. No effect of day or interaction between day and treatment were observed. Cows consuming AF-CON yielded the greatest amount of fat, and CON cows yielded the least fat. The reduced fat yield for CON cows was expected as CON cows produced less milk. Fat yield results from the current study differ from previous research reporting no change[5,11,12,13] or a decrease[15] in fat yield following the administration of AF and clay diets.[5,11,12,13] However, Sulzberger et al[22] reported the opposite, with a tendency for greater fat yields from cows consuming AF diets with no clay compared to control cows. Cows consuming 4C diet yielded the most protein, and cows consuming CON and 4C+AF diets yielded the least protein. Solids yield was greatest in 4C cows and least in CON cows. Cows consuming CON diets tended to yield the least lactose, and cows consuming 4C and 8C+AF diets tended to yield the most lactose. Component yields followed a similar pattern to milk yield, which was expected as the percent of these components was unaffected by treatment. The addition of clay did not appear to negatively affect milk production, and in fact may result in an increase in milk and component yields.

Body Weight, Body Condition, Locomotion, and Respiratory Rate

Cow body weight (BW), condition scores, locomotion scores, and respiratory rate are shown in Table 5. No effect of day or interaction between day and treatment were observed. Cows consuming 4C had the greatest BW followed by 4C+AF. Cows consuming CON diets weighed the least, and AF-CON and AF+8C cows were intermediate. Change in body weight was calculated both weekly and throughout the study, but did not differ across treatments (data not shown). Body condition was similar across treatments. Previous studies reported no change in body weight or body condition score following the addition of AF or clay to the diet;[11,12,22] however, most controlled research studies do not subject cows to AF contaminated diets for the same dose, duration, and condition that cows may be exposed to with naturally occurring AF.[2] There was a tendency for 8C+AF cows to have a greater locomotion score compared to other treatments.

Aflatoxin M_1

A dose response was observed for AFM_1 concentration, secretion, and transfer (Table 6). AFM_1 concentration was below the detection limit (<0.04 ppb) in all 4C and most CON milk samples and was recorded as a not detectable (N/D). One CON sample tested positive for AFM_1 on the second sampling day, so there may have been potential contamination at a feeding. Milk from AF-CON cows contained the greatest concentration of AFM_1. A 23.17% reduction in AFM_1 (ppb) was observed in 4C+AF cows, and a reduction of 45.12% was observed in 8C+AF cows compared to AF-CON cows. Reduction in AFM_1 concentration following administration of a clay adsorbent is well documented.[5,11,12,13,17,22] Previous studies by Maki et al[11,12] and Sulzberger et al[22] reported a greater reduction in AFM_1 concentration as clay dose increased. Daily concentrations of AFM_1 by treatment are reported in Figure 1.

Table 5. Body weight, body condition score, locomotion score, and respiratory rate by treatment with differing levels of aflatoxin and aluminosilicate clay.

	Treatment*						
Item	**CON**	**4C**	**AF-CON**	**4C+AF**	**8C+AF**	**SEM†**	***P*-value**
BW, lb	1517[d]	1587[a]	1524[cd]	1557[b]	1549[bc]	10.7	<0.001
BCS†	3.00	3.04	3.06	3.13	3.03	0.35	0.733
Locomotion[3]	1.25	1.32	1.32	1.21	1.71	0.69	0.057
Respiratory rate, bpm	41.64	39.71	39.29	44.07	38.93	10.19	0.299

* CON = basal TMR with no aflatoxin (AF) or aluminosilicate clay; 4C = basal TMR plus 4 oz aluminosilicate clay; AF-CON = basal TMR plus 113 ppb AF; 4C+AF = basal TMR plus 4 oz aluminosilicate clay and 113 ppb AF; 8C+AF = basal TMR plus 8 oz aluminosilicate clay and 113 ppb AF

† BCS = body condition score; largest standard error of the mean

[a–b]Means within a row with different superscripts differ ($P < 0.05$).

Table 6. Concentration, secretion, and transfer of aflatoxin M_1 (AFM_1) into milk by dietary treatment with differing levels of aflatoxin and aluminosilicate clay.

	Treatment*						
Item	**CON**	**4C**	**AF-CON**	**4C+AF**	**8C+AF**	**SEM‖**	***P*-value**
AFM_1, ppb†	<0.01[d]	N/D[d]	1.64[a]	1.26[b]	0.90[c]	0.38	<0.001
Secretion, µg‡	0.06[d]	0.00[d]	48.04[a]	37.36[b]	28.57[c]	1.46	<0.001
Transfer, %§	n/a	n/a	1.60[a]	1.25[b]	0.95[c]	0.05	<0.001

* CON = basal TMR with no aflatoxin (AF) or aluminosilicate clay; 4C = basal TMR plus 4 oz aluminosilicate clay; AF-CON = basal TMR plus 113 ppb AF; 4C+AF = basal TMR plus 4 oz aluminosilicate clay and 113 ppb AF; 8C+AF = basal TMR plus 8 oz aluminosilicate clay and 113 ppb AF

† N/D = not detected; The detection limit was set at 0.04

‡ Secretion = concentration of AFM_1 in milk ∗ milk yield

§ Transfer = (µg AFM_1 secreted/µg AFB_1 administered) ∗ 100

‖ Largest standard error of the mean

[a–b]Means within a row with different superscripts differ ($P < 0.05$).

Milk from cows consuming the 8C+AF diet remained similar in AFM_1 concentration throughout the experiment. Milk from cows consuming 4C+AF diets was most concentrated in AFM_1 on d 7 and 11 and was least concentrated in AFM_1 on d 5. A dose response reduction of AFM_1 concentration with increasing clay dosage was observed on d 7, 11, and 13. On d 3, 5, and 9 AFM_1 concentration was similar between 8C+AF and 4C+AF diets. Milk from cows consuming AF-CON diets was most concentrated in AFM_1 on d 13 and least concentrated on d 3. Administration of 8 oz of aluminosilicate clay with AF challenge appeared to result in a more consistent reduction of AFM_1 concentration throughout the study.

Secretion of AFM_1 followed a similar pattern to AFM_1 concentration with AF-CON cows secreting the greatest amount of AFM_1. Secretion was calculated using a numerical value of 0.00 for N/D samples. Secretion of AFM_1 averaged 0.00 and 0.06 µg/d for 4C and CON cows, respectively. The presence of AF in the CON group was due to the positive

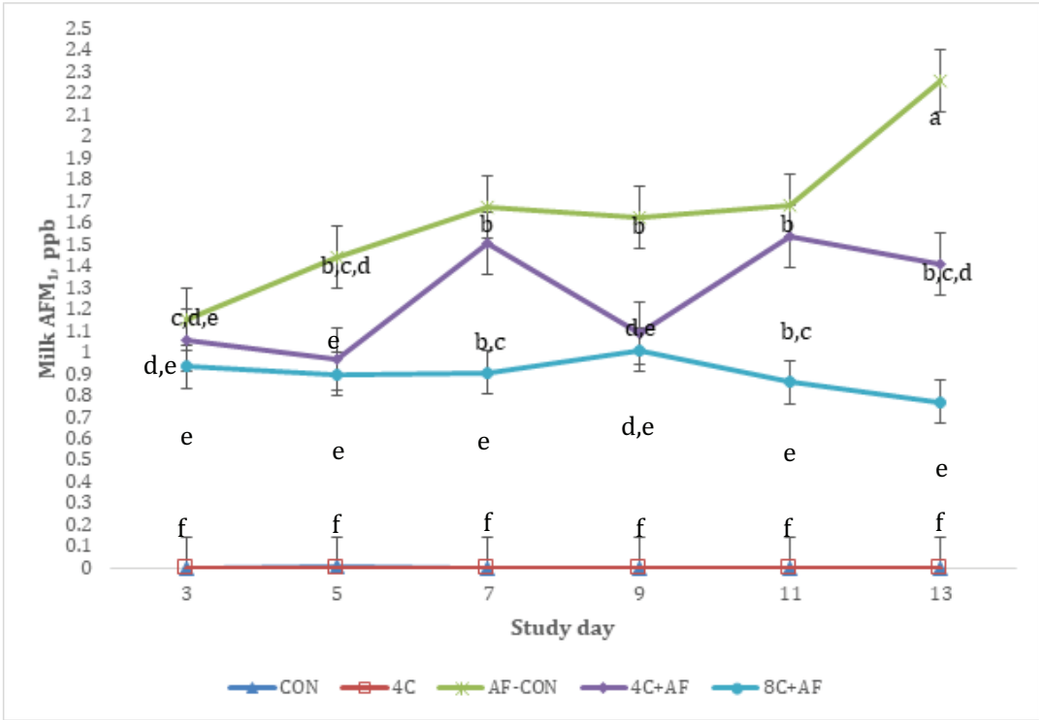

Figure 1. Daily aflatoxin M_1 (AFM_1) concentrations in milk by dietary treatment with differing levels of aflatoxin and aluminosilicate clay. CON = basal TMR with no AF or aluminosilicate clay; 4C = basal TMR plus 4 oz aluminosilicate clay; AF-CON = basal TMR plus 113 ppb AF; 4C+AF = basal TMR plus 4 oz aluminosilicate clay and 113 ppb AF; 8C+AF = basal TMR plus 8 oz aluminosilicate clay and 113 ppb AF.
[a-f] indicates differences among the interaction of treatment and day.

sample reported above. A 22.23% reduction in AFM_1 (μg) secretion was observed in 4C+AF cows, and a secretion reduction of 40.53% was observed in 8C+AF cows compared to AF-CON cows. Sulzberger et al[22] reported linear reductions in AFM_1 secretion to the milk, while studies by Maki et al[11,12,13] reported reduction in secretion, but no difference among clay dose.

Transfer AFM_1 was greatest in AF-CON cows, and a dose-dependent response was observed. A transfer reduction of 21.88 and 40.63% was observed in cows on the 4C+AF and 8C+AF diets, respectively, relative to the AF-CON diet. Similar to secretion of AFM_1, Sulzberger et al[22] reported linear reductions in AFM_1 transfer to the milk, while studies by Maki et al[11,12,13] reported reduction in transfer following the addition of clay, but no difference among clay dose.

Similar patterns have been reported by Maki et al[11,12] when administering a calcium montmorillonite clay adsorbent. In both studies, dietary concentrations of AF and clay were similar to doses used in the current study, but Maki et al[11,12] observed a greater reduction in AFM_1 in milk. It is important to note that AFM_1 concentration and transfer of AF was greater in cows consuming AF with no clay[11,12] compared to AF-CON cows in the current study, so variability in transfer of AF may have resulted in the numerical differences of AFM_1 concentration.

Conclusions

Results from the current study indicate inclusion of aluminosilicate clay successfully reduces the transfer of AFM_1 in the milk of Holstein cows, resulting in the reduction of AFM_1 concentration in the milk. This study suggests that administering the aluminosilicate clay at a dose of 8 oz results in further reduction of AFM_1 transfer compared to 4 oz; however, neither dose reduced milk AFM_1 below the action limit of 0.5 ppb. It is important to note that the dose of AFB_1 was much greater (5.65x) than the action limit in dairy feeds. Further research using either reduced AF doses or increased clay doses may determine the proper dosage of clay to reduce AFM_1 below the action limit. Additionally, further research would provide insight on the effect of lactation performance and determine if the increase in milk performance following the administration of clay can be repeated in subsequent studies.

Endnotes

[a] PMI Additives, Arden Hills, MN
[b] Calan Broadbent Feeding System, American Calan, Northwood, NH
[c] Afimilk Ltd., Kibbutz, Israel
[d] Dairy One, Forage Analysis Laboratory, Ithaca, NY
[e] Thomas Wiley mill, model 4, Thomas Scientific, Swedesboro, NJ
[f] Broad Spectrum Microtabs II™ tablets, Weber Scientific®, Hamilton, NJ
[g] United Federation of DHIA, Radford, VA
[h] Bently FTS Combi, Chaska, MN
[i] SAS® version 9.4, SAS Institute Inc., Cary, NC

Acknowledgements

This project was partially funded by PMI (Arden Hills, MN). We wish to thank the North Carolina Department of Agriculture and Consumer Services Piedmont Research Station for their assistance in data collection and animal care. Kay Russo and Devan Compart are employees of PMI.

References

1. AOAC International. Official Methods of Analysis. 17th ed. Arlington, VA: AOAC Int, 2009.
2. Applebaum RS, Brackett RE, Wiseman DW, Marth EH. Responses of dairy cows to dietary aflatoxin: Feed intake and yield, toxin content, and quality of milk of cows treated with pure and impure aflatoxin. *J Dairy Sci* 1982; 65:1503-1508.
3. Diaz DE, Hagler WM Jr., Hopkins BA, Whitlow LW. Aflatoxin binders. I: In vitro binding assay for aflatoxin B1 by several potential sequestering agents. *Mycopathologia* 2002; 156: 223-226.
4. Diaz DE, Hagler WM Jr., Blackwelder JT, Eve JA, Hopkins BA, Anderson KL, Jones FT, Whitlow LW. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia* 2004; 157:233–241.
5. Kutz RE, Sampson JD, Pompeu LB, Ledoux DR, Spain JN, Vázquez-Añón M, Rottinghaus GE. Efficacy of Solis, NovasilPlus, and MTB-100 to reduce aflatoxin M_1 levels in milk of early to mid-lactation dairy cows fed aflatoxin B_1. *J Dairy Sci* 2009; 92:3959-3963.
6. Food and Drug Administration. Guidance for industry: Action levels for poisonous or deleterious substances in human food and animal feed, 2000. Available at: www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ChemicalContaminantsMetalsNaturalToxinsPesticides/ucm077969.htm#afla. Accessed Jan 30, 2016.
7. Ferguson JD, Galligan DT, Thomsen N. Principal descriptors of body condition score in Holstein cows. *J Dairy Sci* 1994; 77:2695-2703.
8. Galvano F, Galofaro V, Galvano G. Occurrence and stability of aflatoxin M_1 in milk and milk products: a worldwide review. *J Food Prot* 1996; 59:1079-1090.
9. Guo B, Chen ZY, Lee RD, Scully BT. Drought stress and preharvest aflatoxin contamination in agricultural commodity: Genetics, genomics and proteomics. *J Integr Plant Biol* 2008; 50:1281–1291.
10. IARC, International Agency for Research on Cancer, Lione. IARC Monographs on the evaluation of carcinogenic risks to human. Vol. 56. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. 1993.
11. Maki CR, Thomas AD, Elmore SE, Romoser AA, Harvey RB Ramirez-Ramirez HA, Phillips TD. Effects of calcium montmorillonite clay and aflatoxin exposure on dry matter intake, milk production, and milk composition. *J Dairy Sci* 2016; 99:1039-1046.
12. Maki CR, Monteiro APA, Elmore SE, Tao S, Bernard JK, Harvey RB, Romoser AA, Phillips TD. Calcium montmorillonite clay in dairy feed reduces aflatoxin concentrations in milk without interfering with milk quality composition or yield. *Anim Feed Sci and Tech* 2016; 214:130-135.
13. Maki CR, Haney S, Wang M, Ward SH, Rude BJ, Bailey RH, Phillips TD. Calcium montmorillonite clay for the reduction of aflatoxin residues in milk and dairy products. *J Dairy and Vet Sci* 2017; 2:555-587.
14. Ogunade IM, Arriola KG, Jiang Y, Driver JP, Staples CR, Adesogan AT. Effects of 3 sequestering agents on milk aflatoxin M_1 concentration and the performance and immune status of dairy cows fed diets artificially contaminated with aflatoxin B_1. *J Dairy Sci* 2016; 99:6263-6273.

15. Pate RT, Paulus Compart DM, Cardoso FC. Aluminosilicate clay improves production responses and reduces inflammation during an aflatoxin challenge in lactating Holstein cows. *J Dairy Sci* 2018; 101:11421-11434.
16. Purchase IFH, Steyn M, Rinsma R, Tustin RC. Reduction of the aflatoxin M content of milk by processing. *Fd Cosmet Toxicol* 1972; 10:383-387.
17. Queiroz OC, Han JH, Staples CR, Adesogan AT. Effect of adding a mycotoxins-sequestering agent on milk aflatoxin M_1 concentration and the performance and immune response of dairy cattle fed an aflatoxin B_1-contaminated diet. *J Dairy Sci* 2012; 95:5901-5908.
18. Ramos AJ, Hernandez E. In vitro aflatoxin absorption by means of a montmorillonited silicate. A study of absorption isotherms. *Anim Feed Sci Technol* 1996; 62:263-269.
19. Rasooli I, Abyaneh MR. Inhibitory effects of thyme oils on growth and aflatoxin production by *Aspergillus parasiticus. Food Control* 2004; 15:479-483.
20. Shotwell OL, Hesseltine CW, Stubblefield RD, Sorenson WG. Production of aflatoxin on rice. *Appl Microbiol* 1996; 14:425-428.
21. Stoloff L, Trucksess M, Hardin N, Francis OJ, Hayes JR, Polan CE, Campbell TC. Stability of aflatoxin M in milk. *J Dairy Sci* 1975; 58:1789-1793.
22. Sulzberger SA, Melnichenko S, Cardoso FC. Effects of clay after an aflatoxin challenge on aflatoxin clearance, milk production, and metabolism of Holstein cows. *J Dairy Sci* 2017; 100:1856-1869.
23. Van Egmond HP, Jonker MA. Worldwide regulations on aflatoxins—The situation in 2002. *J Toxicol: Toxin Rev* 2004; 23:273-293.
24. Weatherly ME, Pate RT, Rottinghaus GE, Roberti Filho FO, Cardoso FC. Physiological responses to a yeast and clay-based adsorbent during an aflatoxin challenge in Holstein cows. *Anim Feed Sci and Technol* 2018; 235:147-157.
25. Xiong JL, Wang YM, Zhou HL, Liu JX. Effects of dietary adsorbent on milk aflatoxin M_1 content and the health of lactating dairy cows exposed to long-term aflatoxin B_1 challenge. *J Dairy Sci* 2018; 101:8944-8953.
26. Zinpro Corporation, Eden Prairie, MN. Locomotion scoring of dairy cattle. Available at: http://www.zinpro.com/lameness/dairy/locomotion-scoring. Accessed Jun. 23, 2015.