Strategies to Alleviate Aflatoxin Deleterious Effects on Performance, Inflammation, and Oxidative Stress in Dairy Cows

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Take Home Messages

- Feeding clay can help to alleviate the effect of a grain challenge on the rumen environment and ultimately affect the performance of Holstein cows.
- Inclusion of clay products in the diet seems to linearly reduce aflatoxin transfer from the rumen and diet to the milk and feces of mid-lactation Holstein cows.
- Clay's mode of action is commonly associated with its ion-exchanging capacity.
- Injectable trace minerals help alleviate the oxidative stress due to an aflatoxin challenge in dairy cows.

Introduction

All animals are subject to infection from bacteria, viruses, and fungi, but ruminant animals, specifically dairy cattle, can get diseases purely from what they eat (Underwood et al., 2015). For example, the formulation of a diet can cause severe pH changes that can lead to acidosis, or the animal's feed can be contaminated with fungi or bacteria that produce toxins.

Acidosis

Dietary ingredients in dairy cow diets affect animal efficiency and health. To produce milk at maximum efficiency, concentrates are required as a feed choice, but high inclusion of concentrate in total mixed rations (TMR) has gained popularity (Eastridge, 2006). Increasing concentrate-to-forage ratios and more elaborate grain processing in lactating dairy cow diets have been associated with higher milk production (Khorasani and Kennelly, 2001; Yang et al., 2001). However, too much concentrate can challenge the cow's natural buffering capacity and leave the rumen susceptible to drastic drops in pH levels (Shaver et al., 2000). Knowing the diurnal rhythm of rumen pH is crucial to understanding when a cow confronts sub-acute ruminal acidosis (SARA) (Enemark, 2008). The minimum rumen pH fluctuates from 5.4 to 6.6, making it difficult to distinguish what is truly SARA (Duffield et al., 2004; Krause and Oetzel, 2006). Gozho et al. (2005) have defined SARA as when rumen pH is between 5.2 and 5.6 for at least 3 h/day. Cows facing SARA may experience symptoms such as decreased dry matter intake (DMI) and milk production, altered milk composition, diarrhea, and laminitis (Duffield et al., 2004; Gozho et al., 2005; Krause and Oetzel, 2006; Plaizier et al., 2008). Even though SARA is difficult to diagnose, it is estimated to be prevalent in 19 to 26% of early- and mid-lactation dairy cattle (Enemark, 2008; Plaizier et al., 2008).

Toxins

Mycotoxins have always been a feed safety issue because of their harmful effects to ruminant animals when ingested (Campagnollo et al., 2016). There is a plethora of mycotoxins in the world, but most importantly, there has been a rising food safety concern with aflatoxins because of their capability of quickly being transferred into milk (Benkerroum, 2016; Campagnollo et al., 2016; Zhu et al., 2016). There are no known treatments available to treat the toxic effects of aflatoxin, but in the U.S. the Food and Drug Administration has set regulations on the maximum allowable amount of contamination; these are 20 µg/kg aflatoxin B1 (AFB1) in feed and 0.5 µg/kg aflatoxin M1 (AFM1) in milk (Peraica et al., 1999; Giovati et al., 2015).

Aflatoxins are produced by many fungal species in the genus *Aspergillus* and are notorious for infecting 25% of crops in all stages of production, growth, harvest, and storage (FAO, 2004; Kabak et al., 2006; Campagnollo et al., 2016). Various technologies have been developed to reduce the impact of mycotoxins in the dairy industry. Some of these physical and chemical technologies, such as ultraviolet treatments or chemical reactions, are expensive and difficult to implement on farms (Kabak et al., 2006; Zhu et al., 2016). Overall, the addition of clay adsorbents, i.e., smectites, illites and vermiculites, seem to be an easy and inexpensive way to mitigate the effects of mycotoxin on animal health and performance (Kabak et al., 2006; Zhu et al., 2016).

Aflatoxins create vast economic losses to the dairy industry. In terms of animal health, the adverse effects include depressed feed intake, lethargy, reproduction problems, and immune suppression (Whitlow and Hagler, 2005; Abrar et al., 2013; Zhu et al., 2016). Aflatoxins come in many forms, but the most toxic to ruminant animals is AFB1. Anywhere from 0.3% to 6.2% is bio-transformed to AFM1, which is found in tissues or excreted in milk and other fluids (Campagnollo et al., 2016). This bio-transformation has been detected in the serum five minutes after AFB1 dosing and the AFM1 will stay in the cow's system for three to five days after exposure (Mostrom and Jacobsen, 2011; Queiroz et al., 2012; Campagnollo et al., 2016). The AFB1 can be metabolized by many pathways once ingested, but most importantly, it converts into a reactive epoxide (AFB1-8,9-), which binds to DNA, RNA, and proteins to exert toxic effects on the animal (Abrar et al., 2013; Giovati et al., 2015; Campagnollo et al., 2016). Aflatoxins are lipophilic (combines with, or dissolves in fat) molecules, and because the liver is predominantly a lipophilic organ, aflatoxins increase the risk of hepatocellular carcinoma (liver cancer; Mostrom and Jacobsen, 2011; Di Gregorio et al., 2014; Campagnollo et al., 2016). In humans, aflatoxin negatively affects vitamin use and metabolism (Tang et al., 2009; Costanzo et al., 2015). In dairy cows, aflatoxins impair liver activity and suppress immune responses (Bertoni et al., 2008; Queiroz et al., 2012). Aflatoxins are thought to suppress cell-mediated immune responses and can alter the proliferation and differentiation of cells (Corrier, 1991).

When toxins are introduced to the body, the immune system first must identify that a foreign body is present, which occurs via the innate immune system. In the case of mycotoxins, the focus will be placed on those pathways that link together the inflammation markers. The innate immune system works in two ways. The first is to act as a first responder, sending signals for help. The adaptive immunity works to finish the job and keep records to know if or when the invader comes in again. When the innate immune system is working, cytokines (small proteins made by the immune system that act as chemical messengers) are released as a signal to other cells in the body to know when they should perform their job. Cytokines such as TNFα, IFNɣ, and IL-12 may reach all tissues and organs and stimulate a number of responses, but in the liver, they trigger the release of acute phase proteins such as haptoglobin and ceruloplasmin (Bertoni et al., 2008). Yarru et al. (2008) proved that aflatoxins suppress immune function by demonstrating that chicks fed a low dose of aflatoxin had downregulated cytokine IL-6. Aflatoxin also suppresses innate immunity by suppressing the activity of macrophages, and T and B cells (these are different types of white blood cells involved in immune responses) and complement (a complex system of proteins that work together to help eliminate infectious microorganisms; Corrier, 1991). Mycotoxins fed to dairy cows also suppress neutrophil phagocytosis (Korosteleva et al., 2009).

■ Clays

Clay minerals come in contact with humans and animals on a daily basis. Clays can be found in a multitude of environments that involve soils and rocks, and even play an important role in research and development in many scientific fields (Meunier, 2005). Since the 16th century, clays have been discovered and researched and have accumulated a variety of definitions. According to the Clay Minerals Society, the term "clay" refers to a naturally occurring material composed primarily of fine-grained minerals, which is generally plastic at appropriate water contents and will harden when dried or fired (Guggenheim and Martin, 1995). However, the term “clays” can be used in three different ways: for size, for rock, and for minerals. Clay minerals will be the focus of this article. Clay minerals are present in soil, sediments and rock wastes, as well as in the matrix of the Earth’s crust. Thus, it is vital to understand the structure and capacities of the various types of clays.

There are two fundamental criteria to classify clay minerals: the type of layer structure in a ratio of 1:1, 2:1, or 2:1:1, and the type of octahedral sheet, di- or tri-. Each structure has sites where ions can bond to the structure and the number and positions of these bonds can determine its classification. For example, a 1:1 clay structure with dioctahedral orientation is kaolinite (Rouquerol et al., 2014). These structures are tightly bound and cannot hold an interlayer space. The negative charges are located on the outer surfaces and bind by either aluminum or silica. The 2:1 layers are subdivided through an interlayer sheet that can undergo substitution with small atoms such as magnesium, iron, lithium, aluminum, or silica in both the octahedral and tetrahedral layers (Meunier, 2005; Rouquerol et al., 2014). Smectites have many classifications according to the bound cations on the structure. They all have a charge of -0.2 to -0.6 but can be montmorillonite, beidellite, nontronite, saponite, stevensite, or hectorite. Vermiculites have charges of -0.6 to -0.9 but illites have charges or -0.9 to -0.75, the difference between the two being the crystalline features that are either hydrated or not hydrated, respectively (Meunier, 2005). For the purpose of this article, a focus will be placed on the clays with the highest swelling capacity.

An interesting fact about clays in the 2:1 layer category is their capability of “swelling”. When these clays obtain a negative charge through ion substitutions, water and other molecules are able to penetrate the layers causing an increase in the layer spacing, leading to the cations attempting to retain their polar molecule “shell” (Meunier, 2005; Rouquerol et al., 2014). Clays that have the highest swelling capacity result from the nature of the interlayer cation that can form the most water or glycol layers and partial pressures of water or ethylene glycol (Meunier, 2005). This capacity for clay minerals has intrigued the scientific community for years and the use of these clays has been established in various household items. This specific property makes clays great kitty litter. In 1950, kitty litter was introduced to the world of clay adsorbents and has risen to account for 60% of litter products. Sodium bentonites are added for the characteristic clumping feature and added odor control (Murray, 2005). Almost all kinds of paints include clay additives to extend the life of the color and add specific features to paint such as gloss or matte finish (Murray, 2005; Jungang et al., 2012). Ceramic industries are conducting research with clays and different byproducts such as glycerin to make the same infrastructure that bricks have today (Martínez-Martínez et al., 2016). Other items that include clay products are adhesives, cosmetics, floor absorbents, and pharmaceuticals. Medicines use clay products for suspension, capsules, and tablets, and also to treat gastrointestinal disorders (Murray, 2005).

Geophagy (also known as pica), is the craving for substances not commonly regarded as food; i.e., clay, and was first described in historical records as early as 10 BC. Throughout the centuries, speculation on why pica occurred has ranged from mental illness, to helping fetal development, and to treating mineral deficiencies, but mostly for gastrointestinal benefit (Mahaney et al., 2000). In areas and cultures where plants are barely tolerable to eat, such as Guatemala, clay eating is a common practice to mitigate gastrointestinal stress that results from ingestion and allows for broader diets to include plants otherwise considered inedible. Humans are not the only species to ingest clays; animals have been hypothesized to practice geophagy long before humans have (Mahaney et al., 2000). Rats are ubiquitous in consuming clay when experiencing digestive disease or upset (Wiley and Katz, 1998). Slabach et al. (2015) observed mountain goats, known to be deficient in minerals, risking their visibility to predators in order to supplement their nutrients with provided mineral blocks. Eating earthen material such as clay has been thought to adsorb antinutrients and toxins like phenols, bacteria, and their metabolites (Johns and Duquette, 1991; Mahaney et al., 2000). Clays also alleviate symptoms of gastrointestinal stress caused by changes in pH levels, known as acidosis (Krishnamani and Mahaney, 2000; Slabach et al., 2015).

▪ Clay as a Buffer

Understanding how production parameters and rumen, blood, and fecal pH are affected by clay after a grain challenge in Holstein cows deserves attention. Sulzberger et al. (2016) assigned ten multiparous rumen-cannulated Holstein cows at 142 ± 130 (60 to 502) days in milk to one of five treatments in a replicated 5 × 5 Latin square design balanced to measure carryover effects. Periods (21 days) were divided into an adaptation phase (day 1 to 18, with regular total mixed ration (TMR) fed free choice) and a measurement phase (day 19 to 21). Feed was restricted on day 18 to 75% of the average of the TMR fed from day 15 to 17 (dry matter basis), and on day 19 cows received a grain challenge. The challenge consisted of 20% finely ground wheat administered into the rumen via a rumen cannula, based on the average dry matter intake (DMI) obtained on day 15 to 17. Treatments were POS (no clay plus the grain challenge), three different concentrations of clay (0.5, 1, or 2% of dietary DMI) and control (C; no clay and no grain challenge).

Cows developed subacute ruminal acidosis (SARA) when receiving the grain challenge (Gozho et al., 2005). The daily duration in which rumen pH was below 5.6 was less for cows fed C than for those fed POS. These results were expected, because cows fed POS took longer to adjust their rumen environment to the normal pH range compared with cows fed C. Clays have been shown to work as alkalinizers and have great capacity for hydrogen ion exchange at different pH ranges (Yong et al., 1990). The authors reported that illite clay (a type of clay with high concentrations of magnesium and aluminum silicate) had the best buffering capacity in the pH range from 4.5 to 6, similar to the rumen pH range. Additionally, magnesium oxide, when used as a buffer, may increase ruminal outflow, increase the acetate:propionate ratio and improve milk fat tests (Davis, 1979). Earlier reports from Rindsig et al. (1969) concluded that cows fed clay at 5% of dietary DMI increased acetate concentration and decreased propionate concentration in the rumen, leading to significant increases in milk fat percentage. In the Sulzberger et al. (2016) study, a positive linear effect of treatment on rumen pH indicated that clay at 2% of dietary DMI was most efficient in buffering rumen pH and reducing the time spent below rumen pH 5.6 after a grain challenge. Greater concentrations of clay may have allowed for greater buffering capacity (Table 1).

Clay's mode of action is commonly associated with its ability to exchange ions (Yong et al., 1990). Hu and Murphy (2005) reported that buffers used in diets decreased molar proportions of propionate, which in turn increased the acetate:propionate ratio. A higher fat proportion (%) in milk occurs when the acetate to propionate ratio is increased. Cruywagen et al. (2015) used buffered diets and reported a positive influence on milk fat as acetate concentration was increased in the rumen. Interestingly, high-starch diets may increase the bioavailability of mycotoxins by a biochemical mechanism involving a lowered ruminal pH (Pantaya et al., 2016). The study demonstrated that such practice increased the bioavailability of AFB1 and ochratoxin A (OTA) and therefore exacerbated the toxic risk for animals.

▪ Clay as an Adsorbent

Nones et al. (2016) studied the relationship between aflatoxin and stem cell damage in the presence of bentonite adsorbent. They discovered that aflatoxin molecules occupy the interlayer space of the clay structures by forming complexes with the ions contained within the crystalline structure. The adsorbency of a clay mineral depends on the surfactant concentration and the polarity. Thus, greater incorporation of surfactant in clay enhances its adsorbency power, and the more hydrophilic (water loving) the clay, the higher the adsorption with aflatoxin. Many studies have demonstrated the capability of clay minerals to adsorb aflatoxin and decrease AFM1 in milk and alleviate inflammatory suppression. Kutz el al. (2009) reported a 46% reduction in aflatoxin excretion and a 47% reduction in aflatoxin transfer from feed to milk by feeding a silicate clay mixture known as hydrated sodium calcium aluminosilicate (HCAS). A similar aluminosilicate product was used by Queiroz et al. (2012), who found a 45% reduction in milk AFM1 as well as a significant improvement to the immune challenge effect of aflatoxin on haptoglobin. Sodium bentonites have been found to decrease AFM1

Table 1. Least squares means and associated standard errors for rumen, blood, and fecal pH, and blood metabolites response to a grain challenge for cows in positive control with no clay (POS), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and negative control (C) treatments

	Treatment[1]								*P*-value			
	POS	0.5%	1%	2%	C	SEM	Contrasts[2] 1	Contrasts[2] 2	Linear Trt	Quad Trt	TP	Trt × TP
Rumen												
pH	6.03	6.05	6.16	6.20	6.20	0.06	0.003	0.02	0.001	0.53	<0.0001	0.01
pH < 5.6, h [3]	6.36	5.60	4.57	4.88	4.16	1.26	0.32	0.37	0.41	0.55	---	---
Nadir pH	4.94	5.25	5.06	5.12	5.19	0.07	0.06	0.03	0.42	0.20	---	---
AUC, pH × h/d [4]	11.0	7.93	8.56	7.79	7.71	0.80	0.007	0.005	0.03	0.14	---	---
Fecal pH	6.14	6.22	6.18	6.25	6.38	0.04	<0.0001	0.05	0.06	0.72	<0.0001	0.10
Blood												
pH	7.38	7.38	7.39	7.39	7.37	0.01	0.52	0.54	0.32	0.88	0.001	0.57
pCO_2, mmHg	50.5	50.6	49.0	48.8	51.9	1.3	0.42	0.47	0.25	0.83	0.12	0.72
pO_2, mmHg	53.6	52.1	64.4	61.9	49.1	7.48	0.63	0.37	0.20	0.77	0.11	0.28
BE, mmol/L	4.61	4.37	4.39	4.36	4.82	0.48	0.70	0.60	0.71	0.77	<0.0001	0.93
HCO_3, mmol/L	29.7	29.5	29.3	29.4	29.5	0.49	0.80	0.57	0.62	0.69	<0.0001	0.93
tCO_2, mmol/L	31.2	33.5	30.8	30.9	31.6	1.17	0.79	0.71	0.45	0.61	0.2	0.50
O_2 Saturation %	65.4	67.5	69.2	72.1	64.7	3.32	0.86	0.24	0.11	0.89	0.37	0.35
Lactate, mmol/L	1.09	1.13	1.05	0.92	1.13	0.16	0.77	0.45	0.17	0.82	0.06	0.11
Corrected pH[5]	7.36	7.36	7.39	7.37	7.35	0.01	0.76	0.31	0.35	0.25	0.009	0.29
Corrected pCO_2, mmHg[5]	54.0	53.8	52.1	52.2	55.1	1.37	0.54	0.36	0.23	0.66	0.078	0.69
Corrected pO_2, mmHg[5]	58.6	56.7	71.9	67.4	53.8	7.95	0.62	0.35	0.20	0.67	0.11	0.26

[1] Dietary treatments were positive control diet [POS, without clay (0%) and with grain challenge], 0.5% clay diet (0.5%, with 0.5% of the dietary DMI as clay in a top dress), 1% clay diet (1%, with 1% of the dietary DMI as clay in a top dress), 2% clay (2%, with 2% of the dietary DMI as clay in a top dress), and negative control diet (C; without clay and no grain challenge). Top dress vehicle was 500g of grinded corn. Grain challenge: Based on 20% of the average of the DMI of the last 3 d previous to the challenge as finely ground wheat.

[2] Contrasts were 1 = POS (0%) compared with C; 2 = POS (0%) compared with the average of the three treatments (0.5%, 1%, or 2%). Linear and quadratic effects of treatments POS (0%), 0.5%, 1%, and 2%. [3] During the first 24 h. Time points (TP) 0, 4, 8, 12, 16, 20, 24, 48h relative to grain challenge. [4] Negative incremental area under the curve. Baseline rumen pH = 5.6.

[5] Corrected for cow's rectal temperature at time of sampling according to Ashwood et al. (1983).

concentrations by 60.4% (Kissell et al., 2013). Maki et al. (2016b) fed a calcium montmorillonite product that significantly reduced AFM1 excretion in milk.

Sulzberger et al. (2017) used ten multiparous rumen-cannulated Holstein cows (146 ± 69 days in milk) in a replicated 5 × 5 Latin square design balanced to measure carryover effects to study the effects of clay administration on aflatoxin toxicity. Treatments were: no clay plus an aflatoxin challenge (POS), three different concentrations of clay (0.5, 1, or 2% of dietary DMI) plus an aflatoxin challenge, and a control consisting of no clay and no aflatoxin challenge (C). Each period (21 days) was divided in an adaptation phase (day 1 to 14) and a measurement phase (day 15 to 21). From days 15 to 17, cows received an aflatoxin challenge. The challenge consisted of 100 µg of AFB1/kg of dietary DMI, based on DMI on day 12 to 14. The material was fitted into 10-mL gelatin capsules and administered into the rumen through a rumen cannula

Clay feed additives decrease aflatoxin excretion and aflatoxin transfer from feed to milk (Kutz et al., 2009; Kissell et al., 2013; Barrientos-Velazquez et al., 2016; Maki et al., 2016a). Some studies have reported no changes in DMI or milk yield when feeding clay products during an aflatoxin challenge (Queiroz et al., 2012; Maki et al., 2016a,b). However, we (Sulzberger et al., 2017) detected a quadratic treatment effect for DMI and a negative linear treatment effect for milk yield. The small changes in these values tended to cause a difference for 3.5% fat-corrected milk (FCM) and significant differences for 3.5% FCM/DMI and milk/DMI (Table 2). The differences in milk yield could be the result of the cows' metabolism of aflatoxin. Kubena et al. (1998) reported a reduction in feed consumption that adversely affected feed conversion by broiler chickens exposed to aflatoxin.

As clay concentration in the diet increased, AFM1 concentration in milk decreased and the highest reduction occurred in cows receiving 2% clay (Table 3; Sulzberger et al., 2017). Queiroz et al. (2012) reported an increase in aflatoxin excretion in milk at low concentrations of dietary clay inclusion (0.2% of dietary DMI) but when clay was increased to 1% of dietary DMI, aflatoxin excretion decreased by 16%. Maki et al. (2016a) used a clay feed additive at 0.5 and 1% of dietary DMI and found that both percentages decreased AFM1 concentration in milk (51.3 and 69.7%, respectively). In the Sulzberger et al. (2017) study, we detected a significant decrease in AFM1 excretion (µg/d), with reductions of 25% (0.5% clay), 18% (1% clay), and 41% (2% clay), indicating a decrease in the aflatoxin transfer percentage.

Even though clays have been reported to decrease aflatoxins, certain vitamins (A, D, and E) and minerals have decreased in the presence of smectite clays (Tang et al., 2009; Barrientos-Velazquez et al., 2016). In the Sulzberger et al. (2017) study, we detected no significant differences among

Table 2. Least squares means and associated SEM for body weight (BW), body condition score (BCS), and production parameters response of Holstein cows in positive control with no clay (POS, 0%), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and negative control with no clay (C) treatments

	Treatment[1]						*P*-value			
	POS	0.5%	1%	2%	C	SEM	Contrasts[2] 1	Contrasts[2] 2	Linear Trt	Quad Trt
DMI, kg/d	21.54	21.81	22.34	21.43	21.58	0.69	0.94	0.40	0.79	0.05
BW, kg	669.8	665.7	675.1	669.0	667.6	20. 5	0.58	0.97	0.75	0.39
BCS	3.17	3.60	3.13	3.09	2.86	0.28	0.43	0.74	0.53	0.59
Milk yield										
Milk yield kg/d	37.83	37.57	37.28	36.44	38.57	1.49	0.24	0.15	0.02	0.77
3.5% FCM	41.37	38.22	39.32	38.40	42.85	1.81	0.42	0.06	0.20	0.37
ECM	39.62	37.10	37.10	37.11	40.90	1.60	0.38	0.05	0.16	0.40
Milk composition										
Fat, %	4.12	3.68	3.84	3.86	4.19	0.22	0.76	0.15	0.60	0.26
Fat, kg/d	1.54	1.36	1.43	1.40	1.60	0.09	0.54	0.09	0.32	0.34
Protein, %	2.86	2.87	2.90	2.88	2.81	0.05	0.15	0.48	0.50	0.53
Protein, kg/d	1.08	1.07	1.07	1.05	1.07	0.04	0.93	0.49	0.24	0.68
Lactose, %	4.72	4.70	4.70	4.64	4.66	0.04	0.11	0.17	0.03	0.70
Lactose, kg/d	1.78	1.75	1.75	1.69	1.78	0.07	0.99	0.13	0.02	0.73
MUN, mg/dL	10.88	10.54	10.67	10.85	10.38	0.42	0.36	0.65	0.91	0.53
SCC, log transformed	4.85	4.85	4.75	4.85	4.57	0.34	0.21	0.85	0.95	0.67
3.5% FCM/DMI, kg/kg	1.95	1.77	1.75	1.80	1.97	0.09	0.81	0.02	0.22	0.05
ECM/DMI, kg/kg	1.86	1.72	1.69	1.74	1.88	0.07	0.83	0.01	0.19	0.04
Milk/DMI, kg/kg	1.76	1.73	1.67	1.71	1.78	0.06	0.78	0.04	0.14	0.05

[1] Dietary treatments were positive control diet [**POS**, without clay (0%) and with aflatoxin (AF) challenge], 0.5% clay diet (**0.5%**, with 0.5% of the dietary DMI as clay in a top dress), 1% clay diet (**1%,** with 1% of the dietary DMI as clay in a top dress), 2% clay (**2%**, with 2% of the dietary DMI as clay in a top dress), and negative control diet (**C**; without clay and no AF challenge). Top dress vehicle was 500g of ground corn. Aflatoxin challenge: 100 µg AF / kg of DMI of spiked corn, based on average DMI of the last 3 d prior to the challenge.

[2] Contrasts were 1 = POS (0%) compared with C; 2 = POS (0%) compared with the average of the three treatments (0.5%, 1%, and 2%). Linear and quadratic effects of treatments POS (0%), 0.5%, 1%, and 2% clay.

Table 3. Least squares means and associated SEM for aflatoxin in milk, urine, feces, and rumen fluid of Holstein cows in positive control with no clay (POS, 0%), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and negative control (C) treatments

	Treatment[1]						*P*-value			
	POS	0.5%	1%	2%	C	SEM	Contrasts[2] 1	Contrasts[2] 2	Linear	Quad
Milk, AFM_1 (µg/kg)[3]	0.43	0.35	0.30	0.25	0.00	0.06	<0.0001	0.02	0.01	0.44
Milk, AFM_1 d 18 (µg/kg)	0.80	0.58	0.58	0.47	0.00	0.10	<0.0001	0.01	0.02	0.37
AFM Excretion, (µg/d)[4]	27.81	20.83	22.82	16.51	0.00	3.6	<0.0001	0.03	0.02	0.76
AFM Transfer, (%)[5]	1.37	1.01	0.98	0.74	0.00	0.16	<0.0001	0.003	0.002	0.35
Urine, AFM_1 (µg/kg)[6]	6.50	8.60	4.38	5.51	0.01	1.37	0.004	0.80	0.22	0.80
Feces, AFB_1 (µg/kg)[3]	2.78	1.79	1.52	1.48	0.16	0.35	<0.0001	0.01	0.03	0.12
Rumen fluid, AFB_1 (µg/kg)[7]	0.10	0.05	0.02	0.02	0.003	0.03	0.001	0.004	0.01	0.11

[1] Dietary treatments were positive control diet [**POS**, without clay (0%) and with aflatoxin (AF) challenge], 0.5% clay diet (**0.5%,** with 0.5% of the dietary DMI as clay in a top dress), 1% clay diet (**1%,** with 1% of the dietary DMI as clay in a top dress), 2% clay (**2%**, with 2% of the dietary DMI as clay in a top dress), and negative control diet (**C**; without clay and no AF challenge). Top dress vehicle was 500g of ground corn. Aflatoxin challenge: 100 µg AF/ kg of DMI of spiked corn, based on average DMI of the last 3 d prior to the challenge.

[2] Contrasts were 1 = POS (0%) compared with C; 2 = POS (0%) compared with the average of the three treatments (0.5%, 1%, and 2%). Linear and quadratic effects of treatments POS (0%), 0.5%, 1%, and 2% clay.

[3] Samples that were analyzed were collected on d 18 and 21 of each period. TRT × Day $P < 0.0001$ (Milk); TRT × Day $P = 0.0031$ (Feces).

[4] AFM Excretion = AFM1 (µg) concentration in milk on d 18 × Milk yield on d 18 (kg). Calculations were done solely on d 18 to demonstrate the effectiveness at the highest concentration of AFM_1. POS = 35.62 kg, 0.5% = 35.58kg, 1% = 38.77 kg, 2% = 34.91 kg, C = 36.89 kg, SEM = 6.93.

[5] AFM Transfer = (AFM Excretion, µg/d, / AFM Intake, µg/d) × 100

[6] Samples that were analyzed were collected on d 18 of each period.

[7] Samples that were analyzed were collected on d 14, 18, and 21 of each period. TRT × Day $P < 0.0001$.

treatment groups, suggesting that aflatoxin did not alter plasma vitamin A, D, and E concentrations, findings similar to that reported in humans, swine, and chickens (Tang et al., 2009; Trckova et al., 2014; Fowler et al., 2015). In agreement with the results from the Sulzberger et al. (2017) study, Maki et al. (2016b) found no interference with serum vitamin A concentrations when montmorillonite clay was fed to cattle at 18 and 20 g/day.

Ogunade et al. (2016) studied the effects of adding three mycotoxin-sequestering agents (SEQ) to diets contaminated with AFB1 (75 µg/kg of dietary DMI) on reducing milk AFM1 and immune status of dairy cows. Those authors reported that the greater mean fluorescent intensity of staining for CD62L (also called L-selectins) and CD18 (toll-like receptor integrin) on neutrophils [the receptors add the neutrophils to recognize the pathogens (i.e.; bacteria)] of cows fed SEQ1 (yeast cell culture) and SEQ3 (sodium bentonite) diets suggested that these agents altered the migration of neutrophils exposed to aflatoxin. Additionally, feeding the SEQ2 (yeast cell culture mixed with sodium bentonite) diet reduced the inflammatory response caused by the toxin diet (positive control), and the SEQ1 and SEQ3 diets tended to have a similar effect. Similarly, in our experiment, cows fed clay tended to have lower plasma superoxide dismutase plasma concentrations, possibly indicating less oxidative stress.

■ Conclusions

Feeding clay helps alleviate the effects of a grain challenge on the rumen environment and ultimately affects the performance of Holstein cows. Our studies showed that cows fed 0.5, 1, or 2% clay tended to produce more milk and did produce more 3.5% FCM and energy corrected milk than cows not supplemented with clay. Production and physiological parameter responses (e.g., rumen pH) suggest that clay may be an alternative buffer in diets for dairy cows. Additionally, the inclusion of clay products in the diet seems to linearly reduce aflatoxin transfer from the rumen (challenge) to the milk and feces of mid-lactation Holstein cows. Cows that were challenged with aflatoxin and not fed clay had poorer liver function and inflammatory response compared with cows challenged and receiving clay.

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