EFFECTS OF CLAY SUPPLEMENTATION ON RUMEN ENVIRONMENT, METABOLISM, INFLAMMATION, AND PERFORMANCE IN DAIRY COWS.

BY

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THESIS

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ABSTRACT

Oral supplementation of clay has been reported to function as buffer, an adsorbent, and aid in immune function. However, clays come in a variety of structures and each type has their own properties. Clay supplementation effects on rumen, metabolism, and performance have been considered individually among studies. Our objective was to determine the effects of 3 percentages of dietary clay (EcoMix®) supplementation after two different challenges. Challenge one was to induce sub-acute ruminal acidosis by challenge cows intraruminally with ground wheat and the second challenge introduced corn spiked with aflatoxin directly into the rumen.

For the two challenges, 10 multiparous rumen-cannulated Holstein cows [BW (mean ± SD) = 648 ± 12 kg] with 142 ± 130 (60 to 502) DIM were assigned to 1 of 5 treatments in a replicated 5 × 5 Latin square design balanced to measure carryover effects. For the acidosis challenge, periods (21 d) were divided into an adaptation phase (d 1 to 18, with regular TMR fed ad libitum) and a measurement phase (d 19 to 21). Feed was restricted on d 18 to 75% of the average of the TMR fed from d 15 to 17 (DM basis) and on d 19 cows received a grain challenge. The challenge consisted of 20% of the DMI as finely ground wheat administered into the rumen through the rumen-cannula, based on the average DMI obtained on d 15 to 17. For the aflatoxin challenge, 10 multiparous rumen-cannulated Holstein cows [BW (mean ± SD) = 669 ± 20 kg] with 146 ± 69 DIM were assigned to 1 of 5 treatments in a replicated 5 × 5 Latin square design balanced to measure carryover effects. Periods (21 d) were divided in an adaptation phase (d 1 to 14) and a measurement phase (d 15 to 21). From d 15 to 17 cows received an AF challenge. The challenge consisted of 100 µg of Aflatoxin B1 (AFB1)/kg of dietary DMI. The
material was fitted into 10-mL gelatin capsules (TORPAC, Fairfield, NJ) and administered into the rumen through the rumen-cannula based on the average DMI obtained on d 12 to 14.

Treatments for both challenges were: POS, no clay plus grain or AF challenge; three different concentrations of clay (0.5, 1, or 2% dietary DMI) and control (C), no clay and no challenge. Statistical analysis was performed using the MIXED procedure of SAS. Two contrasts CONT1 (POS vs. C), CONT2 (POS vs. the average of 0.5, 1, or 2%) were compared along with the linear and quadratic treatment effects.

Overall, the grain challenge was successful in causing sub-acute ruminal acidosis (rumen, POS = 6.03 ± 0.06, C = 6.20 ± 0.06; fecal, POS = 6.14 ± 0.04 C= 6.38 ± 0.04). Clay supplementation had treatment differences or negative incremental area under the curve, pH below 5.6 × h/d, (0.5% = 7.93 ± 0.83, 1% = 8.56 ± 0.83, and 2% = 7.79 ± 0.83) compared to POS (11.0 ± 0.83). Linear treatment effects on rumen and fecal pH showed an increase in pH for increasing clay percentages in the diet. Cows fed clay tended to have higher milk yield (0.5% = 28.8 ± 3.4 kg, 1% = 30.2 ± 3.4 kg, and 2% = 29.1 ± 3.4 kg, CONT2), have higher 3.5% FCM (0.5% = 29.9 ± 3.5 kg, 1% = 34.1 ± 3.5 kg, and 2% = 33.1 ± 3.4 kg), and higher ECM (0.5% = 29.1 ± 3.3 kg, 1% = 32.8 ± 3.4 kg, and 2% = 31.6 ± 3.3 kg) than cows in POS (27.7 ± 3.4 kg, 28.0 ± 3.4 kg, 27.7 ± 3.3 kg, respectively).

Cows exposed to AF showed effects in plasma to indicate liver damage i.e. a decrease in aspartate aminotransferase (AST) (C = 84.23, POS = 79.17) and glutamate dehydrogenase (GLDH) (C = 91.02, POS = 75.81). There was a downregulation in hepatic expression of HP and STAT3. Cows supplemented with clay showed a significant decrease in AF excretion in milk (AFM1; 0.5% = 20.83 µg/d, 1% = 22.82 µg/d and 2% = 16.51 µg/d) and AF transfer from rumen fluid to milk (AFM1; 0.5% = 1.01%, 1% = 0.98% and 2% = 0.74%) compared with cows in POS.
(AFM1 = 27.81 µg/kg and AF transfer = 1.37%, CONT2). Similarly, average concentration of AFM1 in milk, (0.5% = 0.35 µg/d, 1% = 0.30 µg/d, 2% = 0.25 µg/kg), AFB1 in feces, (0.5% = 1.79 µg/kg, 1% = 1.52 µg/kg, and 2% = 1.48 µg/kg), and AFB1 in rumen fluid, (0.5% = 0.05 µg/kg, 1% = 0.02 µg/kg, 2% = 0.02 µg/kg) were reduced in cows fed clay when compared with POS (0.43 µg/kg, 2.78 µg/kg, 0.10 µg/kg, respectively, CONT2). There also was a linear trend for increased hepatic expression of NFKB1 and TNFA from POS to 2% clay. In conclusion, the effects of SARA and the effects of aflatoxin prove true to their nature, however, clay supplementation appears to alleviate the effects in both challenges.

Key words: Clay, Buffer, SARA, Aflatoxin, Liver, Gene Expression
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CHAPTER I

LITERATURE REVIEW

RUMINANT DIETARY CONCERNS

Ruminant animals have evolved from their sole purpose in agriculture to a broad agricultural research role in areas of genetic engineering, biotechnology, clinical application, and more importantly for agricultural research itself (Underwood, et al. 2015). Agricultural research in the dairy industry has led to improvements in the performance of that animal i.e. reproduction, health, milk yield and components, and even medicinal purposes. Whatever the type of research, animals need to be healthy to be a candidate in order to conduct trials. 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 their feed can be contaminated with fungi or bacteria that produce toxins.

Rumen physiology

Ruminant animals are unlike other mammals when discussing their digestive system because they have a four compartment stomach that gives them the ability to convert plants into animal protein (Demeyer, 1981). One compartment, the abomasum, is similar in function to the
human stomach, but the other compartments, reticulum, rumen, and omasum have non-glandular mucous membranes that act as a protective barrier from the stomach components. Microbes usually reside on the surface of these cells and are have a complex ecosystem that allow fermentation of carbohydrates and nondigestible cellulose to produce VFAs (Krause and Oetzel, 2006; Plaizier et al., 2009; DePeters and George, 2014; Steele et al., 2016). The largest compartment, the rumen, is filled with feed, water, saliva, bacteria, protozoa, Achaea, fungi, and bacteriophages (DePeters and George, 2014; Lean et al., 2014; Dieho et al., 2016). As a general function of the rumen, the animal needs to provide the necessary items to maintain an optimal environment. The animal will eat feed to provide microbes with the necessary nutrients, will provide saliva when chewing, and will drink water. Together, these “ingredients”, will form a perfect mixture of gas, liquid, and solids in an anaerobic environment. The top portion of the rumen contains a gaseous layer that is the result of fermentation which is removed by eructation.

In the middle, there is fibrous material with microorganisms on the surface floating on top of the bottom liquid layer of water, saliva, and soluble feed components, along with the end product of volatile fatty acids (VFAs) (Demeyer, 1981; Prins, 1987). Carbohydrates, fiber, protein, and nitrogen are the primary nutrients introduced to the rumen. The production of VFAs known as acetate and butyrate is favored by the digestion of NDF, pectin, and sugars whereas propionate is
favored by the digestion of sugars, starches, and other soluble carbohydrates. Lactic acid can also build up in with high degradation of sugars and starches (Wester, 2002; Plaizier et al., 2009).

**Rumen pH**

Changes in 1 unit of pH are noted as a 10-fold change in the concentrations of H⁺ and OH⁻ ions present. At pH 7.0, these ions are in equilibrium and in an abundance of H⁺ ions, the concentration drops below 7.0 and becomes acidic, in an abundance of OH⁻ ions the concentrations rises above 7.0 and becomes basic (Shaoyu, 2014). In normal physiology, rumen pH fluctuated from 5.5-7.0 multiple times a day, but as pH decreases toward 5.0, the symbiotic relationship is disrupted (Bravo and Wall, 2016). The rumen pH is extremely important in determining what type of digestion occurs because any change in H⁺ concentration will alter glycolysis and lactate production. Glycolysis is a biochemical pathway that converts glucose into pyruvate which is then converted into the VFA’s as well as CO₂ and CH₄ depending on substrates present, rumen environment, and bacterial populations (Shaoyu, 2014).

**SARA**

Acute and subacute ruminal acidosis (SARA) has been observed in many farms and it has been estimated to cost $1.12 per day per case of SARA, which could end up costing between $500 million to $1 billion dollars annually (Enemark, 2007). The cost associated with SARA varies with severity and farm management. Mostly, the cow decreases in milk production and
efficiency and has long term health effects such as decreased reproductive performance, anorexia, rumenitis, abscesses, and laminitis (Krause and Oetzel, 2006; Enemark, 2007; Underwood, et al. 2015). Acute ruminal acidosis occurs when the pH of the rumen drops below 5.0 and the lactic acid concentrations dramatically rise. This usually occurs in animals who do not have the appropriate amount of lactic acid utilizing bacteria or because their rumen papillae are underdeveloped to absorb such large quantities of VFA’s (Krause and Oetzel, 2006). The different with SARA is that the rumen pH is only slightly depressed, (>5.0<5.6) for more than 3 hours a day.

**Implications of SARA**

As pH gets closer to 5.0, lactate-producing bacteria outperform the lactate-utilizing bacteria, which causes damage to the rumen epithelium (Wester, 2002; Enemark, 2007; Plaizier et al., 2009). Changes in microbial populations in an acidic environment result in a decrease of acetate and an increase in propionate and butyrate. Li et al., (2012) found that a SARA challenged tended to increase the concentration of propionate in the rumen thus, reducing the acetate: propionate ratio. Acetate production is a precursor for a cow’s fat production in milk, so there exists a negative correlation between rumen pH and milk fat (Khorasani et al., 2001; Enemark, 2007). Milk fat production can also be influenced by feeding diets high in fish or vegetable oil to alter the concentrations of ruminal conjugated linoleic acid (CLA). Increases in CLA will cause a
decrease in milk fat due to the altered pathway in rumen biohydrogenation (Hou et al., 2011).

Sub-acute ruminal acidosis also increases the permeability of epithelial cells in the rumen (Steele et al., 2016). This becomes an issue in the rumen because an increase in the number of bacteria produces larger quantities of LPS, a bacterial endotoxin, either naturally or from bacterial death, which leaks into systemic circulation (Wester, L., 2002; Plaizier et al., 2009; Rodríguez-Lecompte et al., 2014). Known immune system cells and proteins i.e. haptoglobin, dendritic cells, and macrophages, are activated as this happens to rid the body of the toxins (Wester, L. 2002; Krause and Oetzel, 2006; Rodrigues-Lecompte et al., 2014; Steele et al., 2016).

**Prevention of SARA**

Major advances in dairy nutrition and health have focused greatly on prevention of SARA due to the major economic losses. Since geophagy has always been around, it was no surprise to see that clay has been used as a feed additive for over 40 years (Rindsig and Schultz, 1969). Buffers may be added to diets low in fiber and high in starch, in times of excessive carbohydrate intake, or when adaptation to highly fermentable diets is poor (Shaver et al., 2000; Krause and Oetzel, 2006; Enemark, 2007). Calsamiglia et al. (2011) explains, SARA is more commonly related to feeding high concentrate diets. It is a matter of altering the fermentation pathways in the rumen and one of the ways to control it is by controlling rumen pH. Few data are available for use of clay buffers in ruminant animals but there have been failed attempts at feeding
vermiculite as a buffer to control or raise ruminal pH (Bringe and Schultz, 1969; Erasmus and Prinsloo, 1989). However, bentonite, also known as smectite clay, have proven to be functional as a buffer. Rindsig et al. (1969) experimented with adding 5% and 10% bentonite clay to lactating cows at risk for SARA. Milk fat production increased for cows receiving clay compared to those that were not, leading to the assumption that ruminal acetate and propionate concentrations were balanced more effectively. More recently, Sulzberger et al. (2016) tested the efficacy of a clay product in mid lactation cows and found a significant increase in rumen fluid pH and a higher nadir pH after a wheat flour challenge.

**TOXINS IN THE DAIRY INDUSTRY**

Not only do some clays have a buffering capacity to raise ruminal pH, they are very well known as a toxin adsorbent due to their charge distributions (Papaioannou et al., 2005). Mycotoxins have constantly been a feed safety issue because of their harmful nature to ruminant animals when ingested (Campagnollo et al., 2016). There are a plethora of mycotoxins in the world, but most importantly, there has been a rising food safety concern with aflatoxins due to 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 United States the FDA has set regulations on the amount of contamination in
feed to 20 µg/kg AFB₁ and in milk to 0.5 µg/kg AFM₁ (Peraica et al., 1999; Giovati et al., 2015). Aflatoxins are produced by many fungi 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). There have been various technologies developed to diminish the impact of mycotoxins in the dairy industry. Some of these physical and chemical technologies such as UV-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 a fairly easy and inexpensive way to mitigate the effects of mycotoxin on animal health and performance (Taylor, 2002; Kabak et al., 2006; Zhu et al., 2016).

**Implications of Aflatoxins**

Aflatoxins create vast economic losses to the dairy industry. In terms of animal health, however, there are even more adverse effects ranging from depressed feed intake, lethargy, reproduction problems, to immune suppression (Whitlow and Hagler, 2005; Abrar et al., 2013; Shrestha and Mridha, 2015; 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 biotransformed to AFM₁, which is found in tissues or excreted in milk and other fluids (Campagnollo et al., 2016). This biotransformation has been detected in the serum 5 minutes after dosing and will stay in the cows
system for 3-5 days after exposure (Mostrom and Jacobsen, 2011; Queiroz et al., 2012; Campagnollo et al., 2016). AFB₁ can be metabolized by many pathways once ingested, but most importantly, it converts into a reactive epoxide (AFB₁-8,9-) via cytochrome P450, which binds to DNA, RNA, and proteins to exert toxic effects on the animal (Abrar et al., 2013; Di Gregorio et al., 2014; Giovati et al., 2015; Campagnollo et al., 2016). Aflatoxins are lipophilic molecules, and because the liver is a predominantly lipophilic organ, they increased risks of hepatocellular carcinoma (Mostrom and Jacobsen, 2011; Di Gregorio et al., 2014; Campagnollo et al., 2016). In humans, aflatoxin has been known to negatively affect vitamin use and metabolism (Tang et al., 2009; Costanzo et al., 2015). Aflatoxins have been proven to impair gene regulation on inflammation processes in chickens (Yarru et al., 2009; Chen et al., 2014). For dairy cows, aflatoxins have been found to impair liver activity and suppress the 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, D. 1991).

**Inflammation effects**

When toxins are introduced to the body the immune system first has to 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 discussed in the study. To recall, the innate immune system works 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 are released as a signal to other cells in the body to know when they should perform their job. Cytokines like TNFa, IFNγ, 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 aflatoxins suppresses immune function by demonstrating that chicks fed a low dose of aflatoxin had downregulated the cytokine IL-6. Aflatoxin has also been shown to suppress innate immunity by suppressing activity of macrophages, T and B cells, and complement (Corrier, 1991). Mycotoxins fed to dairy cows also suppressed neutrophil phagocytosis in a study by Korosteleva et al. (2009).

Prevention and Benefits

Nones et al. (2016) studied the relationship between aflatoxin and stem cell damage in the presence of a 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, the better the incorporation of surfactant in clay gives the higher the adsorbency power, and the more hydrophilic the clay, the higher adsorption with aflatoxin. There are many
studies that have demonstrated the capability of clay minerals to adsorb aflatoxin and decrease AFM$_1$ in milk and alleviate inflammatory suppression. Kutz et 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 aluminosilicates (HCAS). A similar aluminosilicate product was used by Queiroz et al. (2012) and found a 45% reduction in milk AFM$_1$ as well as a significant improvement to the immune challenge effect of aflatoxin on haptoglobin. Sodium bentonites have been found to decrease AFM$_1$ concentrations by 60.4% (Kissell et al., 2012). Maki et al., (2015) fed a calcium montmorillonite product that significantly reduced AFM$_1$ excretion in milk.

**CLAY INTRODUCTION**

Clay minerals widely 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, A. 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. For the purpose of clarification, clay minerals will be the focus of this discussion. Clay minerals are present in soil, sediments, and rock wastes, as well as in the matrix of the Earth’s crust (Mukherjee, S. 2013). Thus, it is vital to understand the structure and capacities of the various types of clays found in the environment.

Clay Structure and Identification

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-. These structures can be seen in Figure 1.1. 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 bound by either Al or Si. The 2:1 layers are subdivided through an interlayer sheet that can undergo substitution with small atoms such as Mg, Fe, Li, Al, or Si in both the octahedral and tetrahedral layers (Meunier, A. 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, A. 2005). Determining classification of various clay minerals can be done through many different techniques. X-ray techniques, such as X-ray diffraction (XRD), expose the target to a beam of electrons, with shorter wavelengths having greater penetration power of the x-rays. Samples can be determined by analytical software and data files that are standard with an XRD machine (Kukherjee and Ghosh, 2013). For the purpose of this study, a focus will be placed on the clays with the highest swelling capacity, the 2:1 layer clays which its structure is represented in Figure 1.1.

**Characteristics, Products and Uses**

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, A. 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, A., 2005). This capacity for clay minerals has intrigued the scientific community for years and their use 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 (Yarnell, A., 2004; Murray, H., 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, H., 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 various items that include clay products are adhesives, cosmetics, floor absorbents, and pharmaceuticals. Medicines use clay products not only for suspension, capsules, and tablets, but also to treat gastro-intestinal disorders (Murray, H 2005).

**Clay’s introduction to diets**

Geophagy, earlier termed pica, is the craving for substances not commonly regarded as food, i.e., clay, was first described in historical records as early as 10 BC. Danford (1982) described the recordings of geophagy throughout earth’s history, each reason differing among cultures. Throughout the centuries, speculation on why pica occurred has ranged from mental illness, to help fetal development, and to treat mineral deficiencies, but mostly for gastrointestinal benefit (Danford, D.E. 1982; 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 plants considered inedible
otherwise (Johns, 1991). 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 Solomon, 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 (Duquette, 1991; Mahaney et al., 2000). Clays also are known to alleviate symptoms of gastrointestinal stress caused by changes in pH levels known as acidosis (Krishnamani and Mahaney, 1999; Slabach et al. 2015).

Overall, the objective of this study is to determine the effects of a bentonite clay product. The objective of this study is to prove that clay supplementation has benefits in health and performance during challenges associated with high concentrates and toxins.
Figure 1.1. Clay Structure showing the ideal structure of a smectite clay in a 2:1 layer. Exchangeable ions represent the various ions that can interact with in the environment.
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CHAPTER 2

Effects of clay after a grain challenge on ruminal, blood, and fecal pH, and milk composition of Holstein cows

ABSTRACT

Oral supplementation of clay has been reported to function as a buffer in dairy cows. However, its effects on rumen, blood, and fecal pH have varied. Our objective was to determine the effects of 3 concentrations of dietary clay (EcoMix®) supplementation after a grain challenge. Ten multiparous rumen-cannulated Holstein cows [BW (mean ± SD) = 648 ± 12 kg] with 142 ± 130 (60 to 502) DIM were assigned to 1 of 5 treatments in a replicated 5 × 5 Latin square design balanced to measure carryover effects. Periods (21 d) were divided into an adaptation phase (d 1 to 18, with regular TMR fed ad libitum) and a measurement phase (d 19 to 21). Feed was restricted on d 18 to 75% of the average of the TMR fed from d 15 to 17 (DM basis) and on d 19 cows received a grain challenge. The challenge consisted of 20% finely ground wheat administered through the rumen-cannula, based on the average DMI obtained on d 15 to 17. Treatments were: POS, no clay plus a grain challenge; three different concentrations of clay (0.5, 1, or 2% dietary DMI) and control (C), no clay and no grain challenge. Statistical analysis was
performed using the MIXED procedure of SAS. Two contrasts **CONT1** (POS vs. C), **CONT2** (POS vs. the average of 0.5, 1, or 2%) were compared along with the linear and quadratic treatment effects (POS, 0.5%, 1%, 2%). Rumen, fecal, and blood pH along with blood metabolites were measured at 0, 4, 8, 12, 16, 20, 24, 36, and 48 h relative to the grain challenge.

Cows in POS had lower rumen pH [(mean ± SE) 6.03 ± 0.06] compared with cows in C (6.20 ± 0.06). Cow in POS had lower fecal pH (6.14 ± 0.04) than C (6.38 ± 0.04). There was a linear treatment effect for rumen pH and fecal pH. Fecal pH (6.22 ± 0.04) was higher for cows that received clay (CONT2) than POS (6.14 ± 0.04). There was a treatment difference (CONT2) for negative incremental area under the curve, pH below 5.6 × h/d, (0.5% clay = 7.93 ± 0.83, 1% clay = 8.56 ± 0.83, and 2% clay = 7.79 ± 0.83) when compared with POS (11.0 ± 0.83). Cows fed clay tended to have higher milk yield (0.5% clay = 28.8 ± 3.4 kg, 1% clay = 30.2 ± 3.4 kg, and 2% clay = 29.1 ± 3.4 kg, CONT2), had higher 3.5% FCM (0.5% clay = 29.9 ± 3.5 kg, 1% clay = 34.1 ± 3.5 kg, and 2% clay = 33.1 ± 3.4 kg), and higher ECM (0.5% clay = 29.1 ± 3.3 kg, 1% clay = 32.8 ± 3.4 kg, and 2% clay = 31.6 ± 3.3 kg) than cows in POS (27.7 ± 3.4 kg, 28.0 ± 3.4 kg, 27.7 ± 3.3 kg, respectively). In conclusion, cows that received clay had higher rumen pH, ECM, FCM, and a trend for higher milk yield than cows in POS.

**Key words**: buffer, clay, rumen pH, grain challenge
INTRODUCTION

Dietary ingredients in feedstuffs used in dairy cow diets affect animal efficiency and health. In order to produce milk at maximum efficiency, concentrates are required as a feed and a high inclusion of concentrate in TMR has gained popularity (Eastridge, 2006). Increasing concentrate to forage ratios and extensive 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 inclusion of concentrates in diets can have a negative impact by challenging the cow’s natural buffering capacity and leaving the rumen susceptible to drastic drops in pH levels (Shaver et al., 2000). Knowledge of the diurnal rhythm of rumen pH is crucial to understanding when a cow encounters sub-acute ruminal acidosis (SARA; Enemark, 2008). The minimum rumen pH physiologically 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) defined SARA to occur when rumen pH is between 5.2 and 5.6 for at least 3 h per day. When a cow faces SARA, she may experience symptoms such as decreased 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 a difficult disease 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). Changes in rumen pH bring about changes in microbial
populations that may lead to a decrease in milk fat percentages (Krause and Oetzel, 2005; Weimer et al., 2010). Additionally, feces of cows experiencing SARA had a lower DM content, longer particles, whole grains, sweet–sour smell, and appeared to be brighter, yellowish, and liquid (Oetzel, 2000; Kleen et al., 2003; Li et al., 2012). These alterations have been theorized to be caused by post-ruminal fermentation in the intestines due to a massive outflow of fermentable carbohydrates from the rumen (Oetzel, 2000; Plaizier et al., 2008). Mixing feed additives (buffers) into the diet for dairy cows has been proven to alleviate adverse health effects caused by SARA (Ghorbani et al., 1989; Shaver et al., 2000; Enemark, 2008; Cruywagen et al., 2015). Buffers have been used primarily to maintain rumen environments with $5.0 < \text{pH} < 7.0$ (Shaver et al., 2000).

Clays, by definition, are the products of silicate rocks which have been subjected to weathering processes for thousands of years (Buckman and Brady, 1969). There are many types of clays that have been used in diets as buffers. Silicates is a specific term to describe clay. The main classification is phyllosilicates, which comprise many subcategories; kaolinite, smectites, chlorites, and micas (Adamis and Williams, 2005). Each term describes the chemical composition of clays; kaolins usually are composed of quartz and mica and have been used for the treatment of digestive problems, and bentonites have been used as enterosorbents (Trckova et al., 2004). Bentonites primarily consist of a mineral called montmorillonite, which has an
articulated layered structure with a net negative charge balanced by cations within the interlayer space. Montmorillonite has an attraction for mono-and divalent ions that bind proteins and nitrogenous compounds to bypass the rumen (Adamis and Williams, 2005; Trckova, et al., 2004; Wester, 2002). Additionally, adding bentonite to corn before ensiling at 0.5% and 1.0% of the wet weight significantly increased pH in the corn silage (Everson et al., 1971).

Understanding how rumen, blood, and fecal pH are affected by clay after a grain challenge in Holstein cows; and clay’s impact on production parameters deserves attention. Therefore, the objectives of this experiment were: 1) to determine the effects of a commercially available clay product after a grain challenge on ruminal, blood, and fecal pH, and milk composition of mid-lactation Holstein cows; and 2) to determine the most appropriate clay concentration to be used in the diet of lactating dairy cows.

MATERIALS AND METHODS

Animals and housing

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (IACUC). The experimental period occurred during September to December, 2014. Cows were housed in tie stalls with sand bedding and ad libitum water and feed access. Mineral salt blocks were available at all times in the alleys.
from the tie stalls to the milking parlor (Big 6 Mineral Salt, North American Salt Company, Overland Park, KS). Diet (TMR) was formulated according to NRC (2001) recommendations.

**Experimental design and grain challenge procedure**

A total of 10 multiparous rumen-cannulated Holstein cows [BW (mean ± SD) = 648 ± 12 kg] with 142 ± 130 (60 to 502) DIM were assigned to 1 of 5 treatments in a replicated 5 × 5 Latin square design balanced to measure carryover effects. Therefore, treatments were arranged so that the carryover effects could be evaluated. Periods (21 d) were divided into an adaptation phase (d 1 to 18, with regular TMR fed ad libitum) and a measurement phase (d 19 to 21). Day 18 had restricted feeding, with cows being offered 75% of the average of the TMR fed on d 15 to 17 (DM basis) and on d 19 a grain challenge occurred. A schematic representation of the experimental phase is shown in Figure 2.1. The grain challenge was similar to the one used for SARA induction proposed by Kmicikewycz and Heinrichs (2014). A grain challenge consisted of finely ground wheat administered via the rumen cannula at a level of 20% of the average DMI obtained on d 15 to 17. Treatments were: POS, no clay plus a grain challenge; three different concentrations of clay (0.5, 1, or 2% dietary DMI) and control (C), no clay and no grain challenge.

The clay used in this experiment was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) and had the respective minerals content (as a percentage of DM):
magnesium = 7.2, silicon = 6.3, aluminum > 5, iron = 6.9, potassium = 0.5, and manganese < 0.1. Ion chromatography (IC) was used to report the presence of other chemical functional groups. The clay’s ionic composition (mg/kg) were: sulfate = 124, chloride = 113, carbonate = 641, nitrate = 97, and phosphate = 2 (Avomeen Analytical Services, Ann Arbor, MI).

All cows were fed the same TMR throughout the trial, fed once daily at 1400h. The daily clay allocation was weighed every day to correlate to the kilograms of TMR offered and equal portions were offered at 0600 and 1400 h. Each portion was mixed with 0.5 kg of ground corn and top dressed on the TMR. Cows in the C and POS treatment groups were given a top dress consisting of 0.5 kg of ground corn only.

**Data Collection and Sampling Procedures**

Samples of feed ingredients and TMR (Tables 1 and 2) were obtained on the first day of each week and analyzed for DM (AOAC, 1995a) by drying for 24 h in a forced-air oven at 110°C. Diet composition was adjusted weekly for changes in DM content of ingredients. The TMR offered and refused from each cow was recorded to determine intake based on weekly DM analyses. Daily DMI was used to calculate daily clay allocation. Total mixed ration samples (a sample of TMR delivered to each cow, n = 10) were taken on d 15 of each period and stored at –20°C until analyzed. Composite samples (3 per period) were analyzed for contents of DM, CP, ADF, NDF, lignin, starch, fat, ash, Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, and S using wet
chemistry methods (Dairy One, Ithaca, NY; [http://dairyone.com/wp-content/uploads/2014/02/Forage-Lab-Analytical-Procedures-Listing-Alphabetical-July-2015.pdf](http://dairyone.com/wp-content/uploads/2014/02/Forage-Lab-Analytical-Procedures-Listing-Alphabetical-July-2015.pdf)). Value for NE\textsubscript{L} was provided by the lab and calculated based on NRC (2001). The physical characteristics of the TMR, based on the Penn State Particle Separator (Kononoff et al., 2003), were determined on the first day of each week.

Cows were milked three times daily at 0400, 1200, and 2200 h. Milk weights were recorded at every milking and samples were obtained from d 15 at each of the corresponding milkings. A preservative was added to each milk sample where they were refrigerated for 3 d (800 Broad Spectrum Microtabs II; D&F Control Systems, Inc., San Ramon, CA). The preserved samples were composited in proportion to milk yield at each sampling and were sent to a commercial laboratory (Dairy Lab Services, Dubuque, IA) to be analyzed for fat, protein, lactose, urea N, total solids, and SCC using mid-infrared procedures (AOAC, 1995b).

Health evaluations were done daily during the last week of each period for the duration of the challenge. Visual assessments were done to monitor general appearance and fecal score. Rectal temperature was measured using a GLA M700 Thermometer (GLA Agricultural Electronics, San Luis Obispo, CA). Respiration rate was recorded by visually watching the cow breathe for 15 s, and heart rate was measured using a stethoscope for 15 s. General appearance was scored using a similar method to Krause et al. (2009): 4 = bright and alert; 3 = depressed; 2
= reluctant to rise; 1 = down cow, will not get up. Fecal scores were allocated on a 1 to 4 scale according to Krause et al. (2009): 1 = runny: liquid consistency, splatters on impact, spreads readily; 2 = loose: may pile slightly and spreads and splatters moderately on impact and setting; 3 = soft: piles up but spreads slightly on impact and settling; 4 = dry: hard, dry appearance, original form not distorted on impact and settling. Body temperature was considered elevated if > 39.4°C, heart rate was considered elevated if > 100 beats/min, respiratory rate was considered abnormal if > 40 breaths/min, general appearance was considered abnormal if ≤ 2, and fecal score was considered abnormal if ≤ 2 (Ireland-Perry and Stallings, 1993; Krause and Oetzel, 2005).

Body weight was measured (Ohaus digital scale, model CW-11, Newark, NJ) and BCS was assigned in quarter-unit increments for each cow weekly (Ferguson et al., 1994). More than one person assigned a BCS score independently at each time of scoring and the average score was used for statistical analysis. Cows were fitted with HOBO pendant® Glogger (Hobo Pendant G Acceleration Data Logger, Onset Computer Corp., Pocasset, MA) positioned laterally on the distal portion of the left hind leg. The activity logger was attached to the leg using vet wrap. Data points were set to record at 60-s intervals. Data collected were used to calculate total lying time, number of lying bouts (number times laid down/24 h), and duration of each lying bout (time
from lying down to standing up), as well as standing time, standing bouts (number times stood/24 h), and duration of each standing bout (time from standing up to lying down).

Rumen fluid (500 mL) was extracted via rumen cannula by pumping a representative sample (from ventral sac, cranial sac, and caudo-ventral blind sac) into a graduated cylinder by use of a syphon for immediate pH measurement with a portable pH meter (AP110 Fisher Scientific, Pittsburgh, PA). Samples were collected at 0, 4, 8, 12, 16, 20, 24, 36, and 48 h relative to the grain challenge (time point zero) on d 19 of each period. At the same time points, fecal samples (approximately 40 g, wet weight) were collected from the rectum of each cow into plastic containers and fecal pH was measured immediately after the collection with the a portable pH meter (AP110 Fisher Scientific, Pittsburgh, PA). Blood samples were collected from the coccygeal vein or artery at 0, 16, 24, and 48 h relative to the grain challenge. Blood samples were collected into tubes containing heparin sulfate and placed on ice. Heparin sulfate tubes were taken to the University of Illinois clinical pathology lab within 10 min off collection for blood gas analysis to measure pH, pCO2, pO2, base excess, HCO3, total CO2 (tCO2), O2 saturation, and lactate (photometric analysis performed according to the lab procedures on the AU680 Chemistry Analyzer, Beckman Coulter, Brea, CA). Additional samples were collected into tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) containing a clot activator for
serum and dipotassium EDTA for plasma. Serum and plasma samples were obtained by centrifugation at 2500 × g for 15 min and stored at −20°C until further analysis.

**Statistical Analyses**

The data were analyzed using the mixed model procedure of SAS (v 9.3; SAS Institute Inc., Cary, NC) to account for carryover effect by the following model

\[ y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + C_m + e_{(ijk)l} , \]

where \( y_{ijklm} \) is the observations for dependent variables; \( \mu \) is the general mean; \( S_i \) is the fixed effect of the \( i^{th} \) treatment sequence; \( A_{(i)j} \) is the random effect of the \( j^{th} \) cow in the \( i^{th} \) sequence; \( P_{(i)k} \) is the fixed effect of the \( k^{th} \) period; \( T_l \) is the fixed effect of the \( l^{th} \) treatment; \( C_m \) is the fixed carryover effect from the previous period (\( C = 0 \), if period = 1); \( e_{(ijk)l} \) is the random error. If carryover effects were not detected, data were analyzed as a replicated Latin square by the following model

\[ y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + D_m + T \times D_{lm} + e_{(ijk)lm} , \]
where \( y_{ijklm} \) is the observation for dependent variables; \( \mu \) is the general mean. \( S_i \) was the fixed effect of the \( i^{th} \) square; \( A_{(i)j} \) is the random effect of the \( j^{th} \) cow in the \( i^{th} \) square; \( P_{(i)k} \) is the fixed effect of the \( k^{th} \) period; \( T_l \) is the fixed effect of the \( l^{th} \) treatment; \( D_m \) is the fixed effect of repeated measurement, which used as TP in pH and day in DMI analysis. The \( T \times D_{lm} \) term is the interaction of treatment and repeated measurement, and the interaction was removed if \( P > 0.3 \); \( e_{ijkl} \) is the random error. The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers (Littell et al., 2002).

Variables were subjected to 5 covariance structures: compound symmetry, autoregressive order 1, autoregressive heterogeneous order 1, unstructured, and Toeplitz. The covariance structure that yielded the lowest corrected Akaike information criterion was compound symmetry and used in the model (Littell et al., 2002).

A log\(_{10}\) transformation was used for the variables milk SCC, lactate, pCO\(_2\), and tCO\(_2\) for better homogeneity of the distribution of residuals. Means shown in tables and graphs for these variables are back-transformed. Orthogonal contrasts were tested using the CONTRAST statement of SAS: 1 = POS (0\% clay) compared with C; 2 = POS (0\% clay) compared with the average of the three clay treatments (0.5, 1, or 2\%) and linear and quadratic effects of treatments POS (0), 0.5, 1, or 2\%. Values reported are least squares means and associated standard errors of the mean. Area under the curve was calculated based on the incremental area method (Cardoso et
al., 2011) with pH = 5.6 as the base line. Residual distribution was evaluated for normality and homoscedasticity. A multivariable logistic mixed model (FREQ procedure) was used for the dichotomized variables (FS, GA, temperature, respiration, and heart rate). The chi-square was computed and is presented. Statistical significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

Diet Composition

The ingredient composition of the diets is detailed in Table 2.1. Analyzed nutrients from the experimental diet are shown in Table 2.2. The physical characteristics of the TMR were (mean ± SD) 4.8 ± 1% on upper sieve, 38.6 ± 3% on middle sieve, 40.7 ± 3% on lower sieve, and 15.8 ± 2% in the pan. Carryover effects were tested and were not present for any variable of interest ($P > 0.45$).

DMI and Lactation Performance

Performance data are shown in Table 2.3. Cows in POS had lower ($P = 0.02$) DMI than cows in C. There was no difference in DMI among cows fed clay (0.5, 1, or 2%) compared with cows in POS. Cows in POS yielded less ($P = 0.009$) milk than cows in C. Cows that received clay (0.5, 1, or 2%) tended to yield more milk ($P = 0.06$), and had higher 3.5% FCM ($P = 0.02$) and
ECM ($P = 0.01$) than cows in POS. Cows that received clay (0.5, 1, or 2%) produced more ($P = 0.05$) milk fat when compared with cows in POS. There was a positive linear treatment ($P = 0.03$) effect on milk fat and an increase in fat (kg/d, $P = 0.05$) when cows were fed clay during a grain challenge. There was a quadratic treatment effect ($P = 0.03$) for milk protein (kg/d), with the C group yielding more protein ($P < 0.01$). Cows that received clay (0.5, 1, or 2%) had a tendency ($P = 0.10$) for a linear treatment effect for 3.5% FCM/DMI, with clay 1% being numerically higher (for every 1 kg of DMI there was 1.99 kg of 3.5%FCM milk produced) than the others.

**Blood Gas and Rumen, Blood, and Fecal pH**

Table 2.4 and Figures 2.2, 2.3, and 2.4 show the results for the blood gas and metabolites, rumen and fecal pH as well as blood pH. Rumen pH was different ($P = 0.003$) between C and POS. Cows that received clay (0.5, 1, or 2) had higher ($P = 0.02$) rumen pH than cows in POS. Time points differed ($P < 0.0001$) for rumen pH, fecal pH, base excess, and blood HCO$_3^-$ as well as blood pH ($P = 0.001$). There was a positive linear treatment ($P = 0.001$) effect on rumen pH, while having a tendency for a positive linear treatment effect ($P = 0.06$) for fecal pH. Nadir rumen pH tended to be lower ($P = 0.06$) for cows in POS than cows in C. Cows that received clay (0.5, 1, or 2%) had higher ($P = 0.03$) nadir rumen pH than cows in POS. Cows in POS spent more time ($P = 0.007$) with rumen pH below 5.6 (AUC) than cows in C. Cows that
received clay (0.5, 1, or 2%) spent less time \((P = 0.005)\) with rumen pH below 5.6 than cows in POS. There was a negative linear treatment \((P = 0.03)\) effect for the time cows spent with rumen pH below 5.6. Fecal pH was different \((P < 0.0001)\) between C and POS. Cows that received clay (0.5, 1, or 2%) had higher \((P = 0.05)\) fecal pH than cows in POS.

**Health and Activity**

No differences were observed for rectal temperature, general appearance, or fecal score among treatments \((P > 0.10)\). Heart rate tended \((P = 0.10)\) to be higher for cows in POS compared with C. Respiratory rate \([(\text{mean} \pm \text{SE}) 37.0 \pm 1.1 \text{ breaths/min}]\) was higher \((P = 0.02)\) for cows that received clay (CONT2) then POS \((34.1 \pm 1.7)\). Table 2.5 is an overview of the activity log of all cows through the trial. Cows that received clay tended to have less lying time \((P = 0.09)\), more standing bouts \((P = 0.09)\), and higher total standing time \((P = 0.09)\) than cows in POS. There was a tendency for a quadratic treatment effect for standing bouts \((P = 0.08)\) and standing time \((P = 0.07)\).

**DISCUSSION**

The aims of this study were to determine the effects of a clay product after a grain challenge on ruminal, blood, and fecal pH, and milk composition of mid-lactation Holstein cows; and to determine the most appropriate clay concentration to be used in the diet of dairy cows.
We postulated that clay administration, in different concentrations, would alleviate the effects of a grain challenge and might impact the rumen or intestinal environment or modulate the acid-base metabolism, thereby affecting DMI, nutrient supply, metabolic responses, and performance in Holstein cows.

Cows in the present study encountered SARA when receiving the grain challenge (Gohzo et al., 2005). Cows in C had less AUC below rumen pH 5.6. These results were expected because cows in POS took longer to adjust their rumen environment to the normal pH range when compared with cows in C (Figure 2.2). Others were also successful in reducing rumen pH after a grain challenge (Krause and Oetzel, 2005; Kmikiewycz and Heinrichs, 2014). Stage of lactation (DIM) seemed not to have interfered with the effectiveness of the grain challenge. In the present study, cows were in a later stage of lactation compared with the aforementioned experiments. The association of SARA with DMI depression, milk yield depression, reduced feed efficiency, rumenitis, diarrhea, laminitis, inflammation, liver abscesses, and high culling and death rates in dairy cattle has been extensively reported by previous authors (Kleen et al., 2003; Al-Zahal et al., 2007; Kmikiewycz and Heinrichs, 2014). In the present study, cows receiving the grain challenge had reduced DMI when compared with cows not receiving the grain challenge (CONT1; Table 2.3)
In the meta-analysis done by Hu and Murphy (2005), rumen pH increased when buffered diets were used compared with unbuffered diets. The higher contents of Mg and Al silicate may have contributed to the buffering capacity of clay in the present study. Clays have been shown to have alkalinizing capacity and have an ability for H+ exchange at different pH ranges (Yong et al., 1990). The authors reported that illite clay (type of clay with high concentration of Mg and Al silicate) had the best buffer capacity in the pH range from 4.5 to 6, similar to the rumen pH range. Additionally, MgO when used as a buffer may function to increase ruminal outflow, which increases the acetate: propionate ratio and improves milk fat test (Davis, 1979).

Earlier reports from Rindsig et al. (1969) concluded that cows being fed clay at 5% (dietary DMI) had increased acetate and decreased propionate in the rumen were associated with significant increases in milk fat percentage. In our study, a positive linear effect of treatment on rumen pH indicated that clay at 2% 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.

Clay’s mode of action is commonly associated with its ion-exchanging capacity (Yong et al., 1990). For instance, clay materials are often employed as backfill or buffer materials for radioactive waste disposal sites because of their ion-exchange properties, low permeability, and easy workability (Kumar and Jain, 2013). Hu and Murphy (2005) reported in a meta-analysis that
buffers used in diets decreased molar proportions of propionate, which in turn increased the acetate: propionate ratio. Cruywagen et al. (2015) used buffered diets and reported that as acetate was increased in the rumen, there was a positive influence on milk fat.

Since clay buffers cause a shift in microbial population to favor acetate in the rumen causing an increase in milk fat, it can be hypothesized that the clay product used in the present study worked in the same way (Rindsig et al., 1969; Ghorbani et al., 1989; Cruywagen et al., 2015). Although VFAs were not the main objective of the study, milk fat was significantly increased for cows receiving clay during a grain challenge. In addition to milk fat increasing, others found increases in milk protein yield when being fed buffered diets. Cows from 246 to 308 DIM of lactation that were fed either 1 kg or 1% sodium sesquicarbonate had increased milk protein yield but there was no effect for cows earlier in their lactation (Tucker et al., 1994; Clark et al., 2009).

Hu and Murphy (2005) reported that, with corn silage based diets, cows ate more when fed sodium bicarbonate, and if corn silage was not the main forage, sodium bicarbonate had no effect on DMI. Others have found similar results in which buffers had little to no effect on milk production or DMI when the cow’s diet was not primarily composed of corn silage (Rearte et al., 1984; Kairenius et al., 2015). Ehrlich and Davison (1997) reported a decrease in DMI when cows were fed 4% sodium bentonite; however, these cows were being fed bentonite mixed with
sorghum grain and not mixed in with the TMR, which may explain the decrease. Unlike the proposed increase in DMI, the present study had no effect on DMI when fed clay, but cows did increase milk yield compared to POS. Cruywagen et al. (2015) found an increase in milk yield for cows fed a mixture of 0.8% sodium bicarbonate and 0.35% limestone. Cows fed clay in the present study tended to increase milk yield and increased milk fat without changing DMI, ultimately causing an increase in 3.5% FCM and ECM. Perhaps these happen in response to the buffering effects discussed above.

Fecal pH has continued to be under-studied by researchers. In 1980, fecal pH was reported to increase when a combination of 1.5% sodium bicarbonate and 0.8% magnesium oxide was fed (Erdman et al., 1980). Results from the present study show a similar increase in fecal pH. Enemark (2008) reported that fecal pH is not normally related to ruminal pH unless starch is able to bypass the rumen and result in hindgut fermentation. If enough carbohydrates were to bypass the rumen and reach the large intestine, cows would experience hindgut acidosis in which the fecal consistency could change to frothy and with mucin casts (Gressley et al., 2011). There were no significant differences in fecal consistency (score) in the present study; however, Li et al. (2012) reported that fecal pH decreased when cows experienced SARA.

In the present study, fecal pH was significantly higher for cows receiving clay during a grain challenge. The addition of clay might have caused changes in post ruminal fermentation
because of reduced outflow of fermentable carbohydrates from the rumen, or binding of fluid in
the intestinal lumen with high osmolality (Oetzel, 2000). On the other hand, Luan et al. (2016)
conducted a similar study but did not find any differences in fecal pH. This could be explained
by the modest reduction in rumen pH in that experiment. Fecal pH and rumen pH patterns were
similar but with a time lag (Figures 2.2 and 2.3). This delay may be related to the amount of
carbohydrates passing the rumen even though fecal appearances did not have any physical
characteristics known for hindgut acidosis (Plaizier et al., 2008).

Blood gas measurements showed that the physiology of the cow was able to maintain
blood pH and its components at constant levels (Dominiczak and Szczepanska-Konkel, 2014). In
the current study, cows fed clay had blood pH values that were closer to the reported normal
range of blood pH at 7.4 (Bigner et al., 1997). Although there were no differences among
treatments, each treatment was different at different time points. From Figure 2.4, the cows in
POS reached the lowest blood pH of all treatment groups about 7.33 at 12 h post grain challenge.
The diet containing clay 1% only reached a blood pH of 7.36 at the same time point and regained
normal values by 24 h. Bigner et al. (1997) conducted a study in which 500 g of sodium
bicarbonate and 343 g of sodium propionate oral treatments raised blood pH. An association
between blood pH and HCO₃ had been noted; however, in our study we did not find any link
between blood pH and clay administration. There was a tendency for a linear increase in O₂
satisfaction percentage (Table 2.4) as clay was included in the diet. According to Karnland et al. (2006) there is a fraction of extractable iron oxides derived from some clay structures, upwards of 3.5%, that were extracted from montmorillonite samples found in Germany, Czech Republic, and India. Therefore, one could hypothesize that the increased O2 saturation could be derived from the increased clay inclusion in the diet. However, the simple fact that cows had a higher respiration rate when fed clay through the challenge phase could have explained the linear effect on blood O2 saturation.

Cows that received clay tended to spend less time lying down, and increased total time spent standing as well as increased number of standing bouts. DeVries et al. (2009) correlated standing time and lying activity with eating behavior. As the risk of acidosis becomes more prominent, the frequency of meals decreases while the duration of meal time increases. In the present study, there were no differences in DMI, however, meal time was not measured.

**CONCLUSIONS**

Feeding clay helped alleviate the impact of a grain challenge on the rumen environment and ultimately affected the performance of Holstein cows. Cows that received 0.5%, 1%, or 2% clay tended to yield more milk, and did yield more 3.5% FCM and ECM than cows in POS. A positive linear treatment effect on rumen pH indicated that clay at 2% was most efficient in
buffering rumen pH and reducing the time spent below rumen pH 5.6 after a grain challenge. In conclusion, production and physiological parameters suggest that clay may be an alternative buffer in diets for dairy cows.

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### TABLES AND FIGURES

**Table 2.1.** Ingredient composition of the lactation diet fed to cows in positive control with no clay (POS), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and negative control (C) treatments throughout the experimental period

<table>
<thead>
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<th>Ingredient</th>
<th>% of DM</th>
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<tbody>
<tr>
<td>Alfalfa hay</td>
<td>8.20</td>
</tr>
<tr>
<td>Corn silage</td>
<td>33.6</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>7.20</td>
</tr>
<tr>
<td>Wet brewers grain</td>
<td>7.49</td>
</tr>
<tr>
<td>Dry ground corn grain</td>
<td>21.55</td>
</tr>
<tr>
<td>Soybean meal, 48%</td>
<td>3.25</td>
</tr>
<tr>
<td>Expeller soybean meal&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.30</td>
</tr>
<tr>
<td>Soy hulls</td>
<td>10.42</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.13</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.35</td>
</tr>
<tr>
<td>Bypass fat&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.87</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.34</td>
</tr>
<tr>
<td>Molasses, sugarbeet</td>
<td>2.05</td>
</tr>
<tr>
<td>Salt (plain)</td>
<td>0.04</td>
</tr>
<tr>
<td>Mineral and vitamin mix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<sup>1</sup> SoyPlus<sup>®</sup> (West Central, Ralston, IA)

<sup>2</sup> Energy Booster 100<sup>®</sup> (Milk Specialties Global, Paris, IL)

<sup>3</sup> Mineral and Vitamin mix was formulated with 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5,000 mg/kg of Cu, 250 mg/kg of I, 40 mg/kg of Co, 150 mg/kg of Se, 2,200 kIU/kg of vitamin A, 660 kIU/kg of vitamin D<sub>3</sub>, and 7,700 IU/kg of vitamin E.
Table 2.2 Mean chemical composition and associated standard deviations for diets fed to cows in positive control with no clay (POS), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and negative control (C) treatments throughout the experimental period

<table>
<thead>
<tr>
<th>Item</th>
<th>Period 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>SD^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>48.4</td>
<td>44.5</td>
<td>47.3</td>
<td>46.4</td>
<td>43.1</td>
<td>2.62</td>
</tr>
<tr>
<td>CP, % of DM</td>
<td>15.8</td>
<td>14.5</td>
<td>15.1</td>
<td>14.8</td>
<td>14.6</td>
<td>0.80</td>
</tr>
<tr>
<td>ADF, % of DM</td>
<td>22.5</td>
<td>22.0</td>
<td>22.2</td>
<td>22.2</td>
<td>24.2</td>
<td>0.40</td>
</tr>
<tr>
<td>NDF, % of DM</td>
<td>32.4</td>
<td>32.7</td>
<td>32.6</td>
<td>34.1</td>
<td>31.6</td>
<td>0.60</td>
</tr>
<tr>
<td>Lignin, % of DM</td>
<td>4.0</td>
<td>3.3</td>
<td>4.1</td>
<td>3.4</td>
<td>3.9</td>
<td>1.20</td>
</tr>
<tr>
<td>Starch, % of DM</td>
<td>27.4</td>
<td>29.9</td>
<td>28.8</td>
<td>28.9</td>
<td>27.4</td>
<td>1.30</td>
</tr>
<tr>
<td>Crude fat, % of DM</td>
<td>5.5</td>
<td>4.9</td>
<td>5.2</td>
<td>4.9</td>
<td>4.4</td>
<td>0.49</td>
</tr>
<tr>
<td>Ash, % of DM</td>
<td>6.54</td>
<td>6.89</td>
<td>6.06</td>
<td>6.38</td>
<td>7.06</td>
<td>0.56</td>
</tr>
<tr>
<td>TDN, % of DM^3</td>
<td>72</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>71</td>
<td>2.12</td>
</tr>
<tr>
<td>NE_L, Mcal/kg of DM^3</td>
<td>1.71</td>
<td>1.71</td>
<td>1.74</td>
<td>1.74</td>
<td>1.66</td>
<td>0.06</td>
</tr>
<tr>
<td>Ca, % of DM</td>
<td>0.71</td>
<td>0.76</td>
<td>0.64</td>
<td>0.66</td>
<td>0.69</td>
<td>0.06</td>
</tr>
<tr>
<td>P, % of DM</td>
<td>0.40</td>
<td>0.30</td>
<td>0.31</td>
<td>0.31</td>
<td>0.35</td>
<td>0.02</td>
</tr>
<tr>
<td>Mg, % of DM</td>
<td>0.22</td>
<td>0.20</td>
<td>0.19</td>
<td>0.20</td>
<td>0.26</td>
<td>0.02</td>
</tr>
<tr>
<td>K, % of DM</td>
<td>1.29</td>
<td>1.16</td>
<td>1.07</td>
<td>1.02</td>
<td>1.41</td>
<td>0.15</td>
</tr>
<tr>
<td>Na, % of DM</td>
<td>0.23</td>
<td>0.26</td>
<td>0.23</td>
<td>0.21</td>
<td>0.22</td>
<td>0.07</td>
</tr>
<tr>
<td>S, % of DM</td>
<td>0.22</td>
<td>0.19</td>
<td>0.19</td>
<td>0.20</td>
<td>0.21</td>
<td>0.01</td>
</tr>
<tr>
<td>Fe, ppm</td>
<td>711</td>
<td>699</td>
<td>522</td>
<td>631</td>
<td>674</td>
<td>325</td>
</tr>
<tr>
<td>Zn, ppm</td>
<td>94</td>
<td>89</td>
<td>84</td>
<td>93</td>
<td>85</td>
<td>5.66</td>
</tr>
<tr>
<td>Cu, ppm</td>
<td>17</td>
<td>17</td>
<td>16</td>
<td>17</td>
<td>17</td>
<td>2.12</td>
</tr>
<tr>
<td>Mn, ppm</td>
<td>90</td>
<td>96</td>
<td>77</td>
<td>86</td>
<td>87</td>
<td>3.51</td>
</tr>
<tr>
<td>Mo, ppm</td>
<td>0.7</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
<td>0.35</td>
</tr>
</tbody>
</table>

¹ Period: made up of 3 consecutive weeks. Cows were fed the TMR ad libitum. Each cow was provided with a mixture of 500g of ground corn and clay at different percentages; 0%, 0.5%, 1%, or 2% of dietary DM. The clay was split into two top dress and fed immediately after feeding and again after 14 h.

² Maximum within period SD.

³ NRC (2001).
Table 2.3 Least squares means and associated standard errors for DMI, BW, BCS, and milk parameters 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

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>POS</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>C</th>
<th>SEM</th>
<th>Contrasts³</th>
<th>P-value</th>
<th>Linear</th>
<th>Quad</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d²</td>
<td>16.97</td>
<td>17.96</td>
<td>16.63</td>
<td>17.52</td>
<td>19.00</td>
<td>1.14</td>
<td>0.02</td>
<td>0.55</td>
<td>0.81</td>
<td>0.84</td>
</tr>
<tr>
<td>BW, kg</td>
<td>643.9</td>
<td>648.4</td>
<td>650.7</td>
<td>648.2</td>
<td>652.1</td>
<td>12.2</td>
<td>0.44</td>
<td>0.54</td>
<td>0.71</td>
<td>0.58</td>
</tr>
<tr>
<td>BCS</td>
<td>3.04</td>
<td>3.23</td>
<td>3.11</td>
<td>3.08</td>
<td>3.24</td>
<td>0.10</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td>0.79</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Milk yield
- Milk yield kg/d: 27.72, 28.77, 30.20, 29.08, 30.58
- 3.5% FCM: 28.24, 29.85, 34.13, 33.09, 30.00
- ECM: 27.71, 29.05, 32.82, 31.56, 29.63

Milk composition
- Fat, %: 3.86, 4.03, 4.20, 4.35, 3.63
- Fat, kg/d: 1.00, 1.08, 1.29, 1.26, 1.04
- Protein, %: 3.01, 3.00, 2.99, 2.92, 3.05
- Protein, kg/d: 0.83, 0.85, 0.89, 0.84, 0.91
- Lactose, %: 4.66, 4.55, 4.67, 4.47, 4.46
- Lactose, kg/d: 1.30, 1.32, 1.43, 1.31, 1.36
- MUN, mg/dL: 12.43, 11.52, 12.18, 11.07, 11.69
- SCC, log₁₀: 4.30, 4.12, 4.26, 4.48, 4.74
- 3.5% FCM/DMI, kg/kg: 1.66, 1.67, 1.99, 1.85, 1.61
- ECM/DMI, kg/kg: 1.62, 1.61, 1.91, 1.76, 1.59
- Milk/DMI, kg/kg: 1.63, 1.57, 1.78, 1.62, 1.63

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 treatment was mixed with 500g of ground 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% clay.

³ TMR was restricted to 75% of d 15 to d 17 average DMI on d18 of each period. These DMI do not include any feed received as part of a grain challenge.
Table 2.4 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

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>POS</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>C</th>
<th>SEM</th>
<th>Contrasts²</th>
<th>Linear Trt</th>
<th>Quad Trt</th>
<th>TP</th>
<th>Trt × TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rumen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.03</td>
<td>6.05</td>
<td>6.16</td>
<td>6.20</td>
<td>6.20</td>
<td>0.06</td>
<td>0.003</td>
<td>0.02</td>
<td>0.001</td>
<td>0.53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pH &lt; 5.6, h³</td>
<td>6.36</td>
<td>5.60</td>
<td>4.57</td>
<td>4.88</td>
<td>4.16</td>
<td>1.26</td>
<td>0.32</td>
<td>0.37</td>
<td>0.41</td>
<td>0.55</td>
<td>---</td>
</tr>
<tr>
<td>Nadir pH</td>
<td>4.94</td>
<td>5.25</td>
<td>5.06</td>
<td>5.12</td>
<td>5.19</td>
<td>0.07</td>
<td>0.06</td>
<td>0.03</td>
<td>0.42</td>
<td>0.20</td>
<td>---</td>
</tr>
<tr>
<td>AUC, pH × h/d ⁴</td>
<td>11.0</td>
<td>7.93</td>
<td>8.56</td>
<td>7.79</td>
<td>7.71</td>
<td>0.80</td>
<td>0.007</td>
<td>0.005</td>
<td>0.03</td>
<td>0.14</td>
<td>---</td>
</tr>
<tr>
<td>Fecal pH</td>
<td>6.14</td>
<td>6.22</td>
<td>6.18</td>
<td>6.25</td>
<td>6.38</td>
<td>0.04</td>
<td>&lt;0.0001</td>
<td>0.05</td>
<td>0.06</td>
<td>0.72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Blood</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.38</td>
<td>7.38</td>
<td>7.39</td>
<td>7.39</td>
<td>7.37</td>
<td>0.01</td>
<td>0.52</td>
<td>0.54</td>
<td>0.32</td>
<td>0.88</td>
<td>0.001</td>
</tr>
<tr>
<td>pCO₂, mmHg</td>
<td>50.5</td>
<td>50.6</td>
<td>49.0</td>
<td>48.8</td>
<td>51.9</td>
<td>1.3</td>
<td>0.42</td>
<td>0.47</td>
<td>0.25</td>
<td>0.83</td>
<td>0.12</td>
</tr>
<tr>
<td>pO₂, mmHg</td>
<td>53.6</td>
<td>52.1</td>
<td>64.4</td>
<td>61.9</td>
<td>49.1</td>
<td>7.48</td>
<td>0.63</td>
<td>0.37</td>
<td>0.20</td>
<td>0.77</td>
<td>0.11</td>
</tr>
<tr>
<td>BE, mmol/L</td>
<td>4.61</td>
<td>4.37</td>
<td>4.39</td>
<td>4.36</td>
<td>4.82</td>
<td>0.48</td>
<td>0.70</td>
<td>0.60</td>
<td>0.71</td>
<td>0.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HCO₃, mmol/L</td>
<td>29.7</td>
<td>29.5</td>
<td>29.3</td>
<td>29.4</td>
<td>29.5</td>
<td>0.49</td>
<td>0.80</td>
<td>0.57</td>
<td>0.62</td>
<td>0.69</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>tCO₂, mmol/L</td>
<td>31.2</td>
<td>33.5</td>
<td>30.8</td>
<td>30.9</td>
<td>31.6</td>
<td>1.17</td>
<td>0.79</td>
<td>0.71</td>
<td>0.45</td>
<td>0.61</td>
<td>0.2</td>
</tr>
<tr>
<td>O₂ Saturation %</td>
<td>65.4</td>
<td>67.5</td>
<td>69.2</td>
<td>72.1</td>
<td>64.7</td>
<td>3.32</td>
<td>0.86</td>
<td>0.24</td>
<td>0.11</td>
<td>0.89</td>
<td>0.37</td>
</tr>
<tr>
<td>Lactate, mmol/L</td>
<td>1.09</td>
<td>1.13</td>
<td>1.05</td>
<td>0.92</td>
<td>1.13</td>
<td>0.16</td>
<td>0.77</td>
<td>0.45</td>
<td>0.17</td>
<td>0.82</td>
<td>0.06</td>
</tr>
<tr>
<td>Corrected pH⁵</td>
<td>7.36</td>
<td>7.36</td>
<td>7.39</td>
<td>7.37</td>
<td>7.35</td>
<td>0.01</td>
<td>0.76</td>
<td>0.31</td>
<td>0.35</td>
<td>0.25</td>
<td>0.009</td>
</tr>
<tr>
<td>Corrected pCO₂, mmHg⁵</td>
<td>54.0</td>
<td>53.8</td>
<td>52.1</td>
<td>52.2</td>
<td>55.1</td>
<td>1.37</td>
<td>0.54</td>
<td>0.36</td>
<td>0.23</td>
<td>0.66</td>
<td>0.078</td>
</tr>
<tr>
<td>Corrected pO₂, mmHg⁵</td>
<td>58.6</td>
<td>56.7</td>
<td>71.9</td>
<td>67.4</td>
<td>53.8</td>
<td>7.95</td>
<td>0.62</td>
<td>0.35</td>
<td>0.20</td>
<td>0.67</td>
<td>0.11</td>
</tr>
</tbody>
</table>

1. Treatment: POS, 0.5%, 1%, 2%, C
2. Contrasts: 2, Linear, Quad, TP, Trt × TP
3. P-value
Table 2.4 continued

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% clay.

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).
Table 2.5 Least squares means and associated standard errors for standing and lying behavior 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 throughout the experimental period

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment¹</th>
<th>POS</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>C</th>
<th>SEM</th>
<th>Contrasts²</th>
<th>Linear</th>
<th>Quad</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trt</td>
<td>Trt</td>
<td></td>
</tr>
<tr>
<td>Lying bouts, no.³</td>
<td></td>
<td>24.2</td>
<td>21.0</td>
<td>23.4</td>
<td>23.62</td>
<td>24.06</td>
<td>5.37</td>
<td>0.96</td>
<td>0.49</td>
<td>0.88</td>
<td>0.49</td>
</tr>
<tr>
<td>Lying duration, min</td>
<td></td>
<td>50.6</td>
<td>55.7</td>
<td>51.6</td>
<td>49.84</td>
<td>52.18</td>
<td>5.59</td>
<td>0.76</td>
<td>0.69</td>
<td>0.65</td>
<td>0.48</td>
</tr>
<tr>
<td>Lying time, min</td>
<td></td>
<td>821.5</td>
<td>750.3</td>
<td>785.5</td>
<td>763.5</td>
<td>787.2</td>
<td>37.8</td>
<td>0.35</td>
<td>0.09</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>Standing bouts, no.³</td>
<td></td>
<td>9.3</td>
<td>10.3</td>
<td>10.29</td>
<td>9.92</td>
<td>10.07</td>
<td>0.64</td>
<td>0.21</td>
<td>0.09</td>
<td>0.56</td>
<td>0.08</td>
</tr>
<tr>
<td>Standing duration, min</td>
<td></td>
<td>73.9</td>
<td>73.4</td>
<td>72.3</td>
<td>73.18</td>
<td>70.42</td>
<td>7.26</td>
<td>0.29</td>
<td>0.80</td>
<td>0.85</td>
<td>0.76</td>
</tr>
<tr>
<td>Standing time, min</td>
<td></td>
<td>657.6</td>
<td>725.4</td>
<td>700.4</td>
<td>681.7</td>
<td>663.8</td>
<td>39.5</td>
<td>0.84</td>
<td>0.09</td>
<td>0.83</td>
<td>0.07</td>
</tr>
</tbody>
</table>

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% clay.

³ No. = number of bouts/24 h.
Figure 2.1 Schematic design of each experimental period and its sampling time points. Milk = composite milk sample, R = rumen sample, F = fecal sample, B = blood sample. Similar diets were fed the entire period prior to the grain challenge.
Figure 2.2 Least squares means and associated standard errors for rumen pH response to a grain challenge (0 h) 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 from 0 to 48 h (time points) relative to a grain challenge. Treatment: $P = 0.003$; CONT1: $P = 0.02$; CONT2: $P = 0.001$; linear treatment effect: $P = 0.53$; time point: $P < 0.0001$, treatment $\times$ time point: $P = 0.01$. 
Figure 2.3 Least squares means and associated standard errors for fecal pH response to a grain challenge (0 h) 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 from 0 to 48 h (time points) relative to a grain challenge. Treatment: \( P < 0.01 \); CON1: \( P = 0.05 \); CON2: \( P = 0.06 \); linear treatment effect: \( P = 0.72 \); time point: \( P < 0.0001 \), treatment \( \times \) time point: \( P = 0.10 \).
Figure 2.4 Least squares means and associated standard errors for blood pH response to a grain challenge (0 h) 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 from 0 to 48 h (time points) relative to a grain challenge. Treatment: $P = 0.52$; CON1: $P = 0.54$; CON2: $P = 0.32$; linear treatment effect: $P = 0.88$; time point: $P = 0.001$, treatment × time point: $P = 0.57$. 

![Graph showing blood pH response to a grain challenge for different treatments over time]
REFERENCES


CHAPTER 3

Effects of clay after an aflatoxin challenge on aflatoxin clearance, milk production, and metabolism of Holstein cows

ABSTRACT

Oral supplementation of clay to dairy cattle has been reported to reduce toxicity of aflatoxin (AF) in contaminated feed. The objective of this study was to determine the effects of 3 concentrations of dietary clay supplementation in response to an AF challenge. Ten multiparous rumen-cannulated Holstein cows [BW (mean ± SD) = 669 ± 20 kg and 146 ± 69 DIM], were assigned to 1 of 5 treatments in a randomized replicated 5 × 5 Latin square design balanced to measure carryover effects. Periods (21 d) were divided in an adaptation phase (d 1 to 14) and a measurement phase (d 15 to 21). From d 15 to 17 cows received an AF challenge. The challenge consisted of 100 μg of Aflatoxin B₁ (AFB₁)/kg of dietary DMI. The material was fitted into 10-mL gelatin capsules (TORPAC, Fairfield, NJ) and administered into the rumen through a rumen-cannula based on the average DMI obtained on d 12 to 14. Treatments were: POS, no clay plus an AF challenge; three different concentrations of clay (0.5%, 1%, or 2% of dietary DMI) and control (C), no clay and no AF challenge. Statistical analysis was performed using the MIXED procedure of SAS. Two contrasts CONT1 (POS vs. C) and CONT2 (POS vs. the average of 0.5%, 1%, or 2%) were compared along with the linear and quadratic treatment effects (POS, 0.5%, 1%, 2%). Cows supplemented with clay had lower AF excretion in milk (AFM₁; 0.5% = 20.83 μg/d, 1% = 22.82 μg/d, and 2% = 16.51 μg/d) and AF transfer from rumen fluid to milk (AFM₁; 0.5% = 1.01%, 1% = 0.98%, and 2% = 0.74%) compared with cows in POS (AFM₁ = 27.81 μg/d and AF transfer = 1.37%, CONT2). Similarly, concentration of AFM₁ in milk (0.5% = 0.35 μg/kg, 1% = 0.30
µg/kg, 2% = 0.25 µg/kg), AFB₁ in feces (0.5% = 1.79 µg/g, 1% = 1.52 µg/kg, 2% = 1.48 µg/kg), and AFB₁ in rumen fluid (0.5% = 0.05 µg/kg, 1% = 0.02 µg/kg, 2% = 0.02 µg/kg) were reduced in cows fed clay when compared with POS (0.43 µg/kg, 2.78 µg/kg, 0.10 µg/kg, respectively, CONT2). Cows supplemented with clay had lower 3.5% FCM (0.5% = 38.2 kg, 1% = 39.3 kg, 2% = 38.4 kg, SEM = 1.8) than cows in POS (41.3 kg; SEM = 1.8; CONT2). Plasma superoxidase dismutase (SOD) concentration tended to be lower for cows fed clay in the diet (0.5% = 2.16 U/mL, 1% = 1.90 U/mL, 2% = 2.3 U/mL; SEM = 0.3) than cows in POS (2.72 U/mL; CONT2). Additionally, when cows were exposed to AF without clay in the diet, plasma concentrations of aspartate aminotransferase (AST) decreased from 84.23 (C) to 79.17 (POS) and glutamate dehydrogenase (GLDH) decreased from 91.02 (C) to 75.81 (POS). In conclusion, oral supplementation of clay reduced the transfer of AF from the rumen to milk and feces.

**Key words:** clay, aflatoxin, milk, urine
INTRODUCTION

Each year, USDA, CSREES, and FDA spend approximately US$ 31.1 million in combined efforts for mycotoxin research (Robens and Cardwell, 2005). Aflatoxins (AF) are most commonly found on corn, peanuts, and cottonseed. This mycotoxin is produced by *Aspergillus flavus* and *A. parasiticus* and, when ingested, one of the AF derivatives (AFB$_1$) is bio-transformed into a toxic secondary metabolite (aflatoxin M$_1$; AFM$_1$). Both AFB$_1$ and AFM$_1$ are carcinogenic; however, AFM$_1$ is the most toxic secondary metabolite that is secreted into milk and is classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2002). The AFM$_1$ represents a food safety risk to humans (Plasencia, 2005; Whitlow and Hagler, 2005; Gallo et al. 2015). The production of AF is influenced by several environmental factors including temperature and humidity.

Aflatoxins are estimated to affect 25% of all agricultural commodity crops (FAO, 2004). Governmental research efforts have found a multitude of preventative measures that include pre-harvest and post-harvest strategies. Before harvesting, studies have focused on selecting seed varieties for *Aspergillus sp.* resistance or applying crop rotations to plants that are not susceptible to *Aspergillus sp.* Crop rotations work to diminish the infectious spores left in the soil (Betrán et al., 2005; Kabak et al., 2006). It has been well documented that applying chemical agents to harvested feed before ensiling can also prevent fungal growth (Guo et al, 2005; Kabak et al., 2006). Whichever method is applied, the FDA has regulated AF concentrations in milk and in feed to be a maximum of 0.5 ppb and 20 ppb in the U.S., respectively (Peraica et al., 1999). However, AF is resilient and continues to be problematic for cattle producers. Acute exposure to aflatoxins cause health problems; inappetance, lethargy, and reproductive disorders. Chronic exposure reduces feed efficiency and milk production, can cause jaundice, and interferes with
vaccine-induced immunity. All in all, aflatoxins are responsible for immunosuppression as well as carcinogenic effects on the liver (Whitlow and Hagler, 2005; Shrestha and Mridha, 2015). According to Abrar et al. (2013) the toxicity of aflatoxins originates from the generation of enzymatic intracellular reactive oxygen species, CYP450, that ultimately biotransforms AFB$_1$ leading to the aflatoxin product binding to DNA, RNA, and proteins.

Various types of adsorbents are described by Kolosova and Stroka (2012). The main characteristic of these materials is the capacity they have to exchange ions and reduce the mycotoxins bioavailability to the cow (Carson and Smith, 1983; Trckova et al., 2004; Kabak et al., 2006; Karnland et al. 2006). Kaolinite, smectite, chlorites, and micas are groups of silicates; clay based materials. Montmoillonites are a class of the smectite clay group which has three-layer structures that allow for internal absorption of mono- and divalent ions into each interlayer sheet. Smectites have a wide range of commercial uses and have been reported to adsorb heavy metals, bacteria, and toxic anti-nutritive agents, such as AF (Trckova et al., 2004).

Different types of adsorbents have been studied to act as potential binding agents for aflatoxins. Kutz et al. (2009) and Queiroz et al. (2012) fed cows a TMR mixture with aflatoxin and used hydrated sodium calcium aluminosilicates (HSCAS), a clay material, and a modified yeast cell culture (MTB-100). The HSCAS reduced AFM$_1$ concentrations by 45 to 48%; however, the MTB-100 only reduced AFM$_1$ by 4% in both studies. Maki et al. (2016a) performed a similar study using an improved product that was calcium montmorillonite clay. This product reduced AFM$_1$ transfer into milk from 1.07% to 0.52%. Xiong et al. (2015) reported that the transfer of AFM$_1$ from the TMR to milk was reduced when Solis Mos (Novus International Inc., St. Charles, MO) was added to a TMR contaminated with aflatoxin B$_1$ when compared with the control (AFB$_1$; 0.46 vs. 0.56%, respectively).
Understanding how rumen, milk, feces, and urine are affected by clay after an aflatoxin challenge in Holstein cows and its impact on production parameters deserves attention. Therefore, the objectives of this experiment were: 1) to determine the effects of a commercially available clay product in response to an aflatoxin challenge on blood chemistry, ruminal, milk, and feces aflatoxin concentrations (i.e., transfer), and milk composition of mid-lactation Holstein cows; and 2) to determine the most appropriate clay concentration to be used in the diet of lactating dairy cows.

MATERIALS AND METHODS

Animal care and Housing

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee. The experimental period occurred from October, 2014 to January, 2015. Cows were housed in tie stalls with sand bedding and ad libitum feed and water access. Diet (TMR) was formulated according to NRC (2001) recommendations.

Experimental Design and Aflatoxin Challenge Procedure

A total of 10 multiparous rumen-cannulated Holstein cows [BW (mean ± SD) = 669 ± 20 kg; DIM = 146 ± 69] were assigned to 1 of 5 treatments in a replicated 5 × 5 Latin square design balanced to measure carryover effects. Therefore, treatments were arranged so that the carryover effects could be evaluated. Periods (21 d) were divided in an adaptation phase (d 1 to 14) and a measurement phase (d 15 to 21). From d 15 to 17 cows received an AF challenge. The aflatoxin challenge was similar to the one proposed by Kutz et al. (2009). Dietary aflatoxin was obtained from the Veterinary Medical Diagnostic Laboratory, College Veterinary Medicine, University of
Missouri, Columbia, and consisted of *Aspergillus parasiticus* (NRRL-2999) culture material containing 102 mg/kg of AFB1, 3.5 mg/kg of AFB2, 35 mg/kg of AFG1, and 0.9 mg/kg of AFG2. The challenge consisted of 100 μg of AFB1/kg of dietary DMI via 10-mL gelatin capsules (Torpac, Fairfield, NJ) administered into the rumen through a rumen-cannula based on the average DMI obtained on d 12 to 14. Treatments were: POS, no clay plus an AF challenge; three different concentrations of clay (0.5%, 1%, or 2% of dietary DMI) and control (C), no clay and no AF challenge.

The clay used in this experiment was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) and had the following composition (percentage of DM), respectively: magnesium = 7.2%, silicon = 6.3%, aluminum > 5%, iron = 6.9%, potassium = 0.5%, and manganese < 0.1%. Ion chromatography (IC) was used to report the presence of other chemical functional groups. The clay’s ions composition (PPM) are: sulfate = 124, chloride = 113, carbonate = 641, nitrate = 97, and phosphate = 2 (Avomeen Analytical Services, Ann Arbor, MI). Additionally, the clay was analyzed by x-ray diffraction which indicated the presence of vermiculite, nontronite, and montmorillonite (Frederick Seitz Materials Research Laboratory; University of Illinois at Urbana-Champaign).

All cows were fed the same TMR throughout the trial once daily at 1400h. The daily clay inclusion was weighed after each feeding to correlate to kilograms of TMR offered. The top dress was split into two equal portions that were mixed with 0.5 kg of ground corn and one clay allocation was offered at 0600 and the second was offered at 1400 h. Cows in the C and POS treatment groups were given a top dress consisting of 0.5 kg of ground corn only.

**Data Collection and Sampling Procedures**
Samples of feed ingredients and TMR (Tables 3.1 and 3.2) were obtained on the first day of each week and analyzed for DM (AOAC, 1995a) by drying for 24 h in a forced-air oven at 110°C. Diet composition was adjusted weekly for changes in DM content of ingredients. The TMR offered and refused from each cow was recorded to determine intake based on weekly DM analyses. Daily DMI was used to calculate daily clay allocation. Total mixed ration samples (a sample of TMR delivered to each cow, n = 10) were taken on d 15 of each period and stored at −20°C until analyzed. Composite samples (3 per period) were analyzed for contents of DM, CP, ADF, NDF, lignin, starch, fat, ash, Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, and S using wet chemistry methods (Dairy One, Ithaca, NY; [http://dairyone.com/wp-content/uploads/2014/02/Forage-Lab-Analytical-Procedures-Listing-Alphabetical-July-2015.pdf](http://dairyone.com/wp-content/uploads/2014/02/Forage-Lab-Analytical-Procedures-Listing-Alphabetical-July-2015.pdf)). Values for NE\textsubscript{L} were provided by the lab and calculated based on NRC (2001). Additionally, one composite sample per treatment and period (n = 25) were stored at −20°C until they were sent to the Veterinary Medical Diagnostic Laboratory, College Veterinary Medicine, University of Missouri, Columbia, to be analyzed for aflatoxin concentrations. The physical characteristics of the TMR, based on the Penn State Particle Separator (Kononoff et al., 2003), were determined on the first day of each week.

Cows were milked 3 times daily at 0400, 1200, and 2200h. Milk weights were recorded at every milking and samples were obtained at each milking from every day during the last week of the period. A preservative (800 Broad Spectrum Microtabs II; D&F Control Systems, Inc., San Ramon, CA) was added to the samples taken on d 15 and preserved samples were stored in a refrigerator for 3 d when they were composited in proportion to milk yield and sent to a commercial laboratory (Dairy Lab Services, Dubuque, IA) to be analyzed for contents of fat, true protein, MUN, lactose, total solids, and for somatic cell count (SCC) using mid-infrared
procedures (AOAC, 1995b). In addition, the appearance and disappearance of the aflatoxin excreted in milk was tested at each milking daily during the last week of the period, d 15 to 21, with the use of a SNAP test (SNP; IDEXX, city and state of the company). Milk samples on d 18 and 21 were stored at −20°C until they were sent to the University of Missouri laboratory to be analyzed for AFM$_1$ and AFB$_1$ concentrations by HPLC with fluorescence detection methods as described in depth by Kutz et al. (2009).

Rumen fluid (500 mL) was extracted via rumen cannula by pumping a representative sample (from ventral sac, cranial sac, and caudo-ventral blind sac) into an Erlenmeyer flask through a syphon on d 15, 18, and 21. Urine samples (60 mL) were collected by manually stimulating urination on d 18. Fecal samples (400 g, wet weight) were collected directly from the cow’s rectum on d 18 and 21. Rumen fluid, urine, and fecal samples were stored at −20°C until they were sent to the Veterinary Medical Diagnostic Laboratory, College Veterinary Medicine, University of Missouri, Columbia, to be analyzed for AFB$_1$ and AFM$_1$ concentrations. Prior to analysis, feces were thawed, dried in a forced air oven at 55 °C for 72 h, then samples were ground in a Thomas-Wiley laboratory mill with a 1-mm screen.

Blood was sampled from the coccygeal vein on d 15 to 21 (n = 7) of wk 3 of each period from each cow (BD Vacutainer; BD and Co., Franklin Lakes, NJ). Serum and plasma samples were obtained by centrifugation of the tubes at 2,500 × g for 15 min at 4°C and stored at −20°C for further analysis. Serum samples were sent to the University of Illinois Veterinary Diagnostic Laboratory to be analyzed for bovine chemistry profiles (creatinine, BUN, total protein, albumin, globulin, albumin/globulin, calcium, phosphorus, sodium, potassium, sodium/potassium, chloride, glucose, alkaline phosphate total, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total bilirubin, creatine phosphokinase (CPK), cholesterol total,
glutamate dehydrogenase (GLDH), TCO2, Mg, and anion gap) using the AU680 Beckman Coulter analyzer (http://vetmed.illinois.edu/vet-resources/veterinary-diagnostic-laboratory/clinical-pathology/). Plasma samples (EDTA) were sent to Michigan State University Diagnostic Center for Population and Animal Health to be analyzed for Vitamins A and E (Waters® ACQUITY UPLC® System), vitamin D (radioimmunoassay; RIA), and cholesterol (colorimetric automated analyzer) (https://www.animalhealth.msu.edu/). Commercially available kits were used to analyze heparinized plasma samples for superoxidase dismutase (SOD) activity and total oxidized glutathione (GSH). Plasma superoxidase dismutase activity was assessed using Superoxidase Dismutase Assay kit in which the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine were measured (Cayman Chemical, Ann Arbor, MI) and GSH was measured using the Glutathione Assay kit with an enzymatic recycling method, using glutathione reductase for the quantification of GSH (Cayman Chemical, Ann Arbor, MI); following manufacturer’s instructions.

Health evaluations were done daily during the last week of each period for the duration of the challenge. Visual assessments were done to monitor general appearance and fecal score. Rectal temperature was measured using a GLA M700 Thermometer (GLA Agricultural Electronics, San Luis Obispo, CA). Respiration rate was recorded by visually watching the cow breathe for 15s, and heart rate was measured using a stethoscope for 15s. General appearance was scored using a similar method to Krause et al. (2009): 4 = bright and alert; 3 = depressed; 2 = reluctant to rise; 1 = down cow, will not get up. Fecal scores were allocated on a 1 to 4 scale according to Krause et al. (2009): 1 = runny: liquid consistency, splatters on impact, spreads readily; 2 = loose: may pile slightly and spreads and splatters moderately on impact and setting; 3 = soft: piles up but spreads slightly on impact and settling; 4 = dry: hard, dry appearance,
original form not distorted on impact and settling. Body temperature was considered elevated if > 39.4°C, heart rate was considered elevated if > 100 beats/min, respiratory rate was considered abnormal if > 40 breaths/min, general appearance was considered abnormal if ≤ 2, and fecal score was considered abnormal if ≤ 2 (Ireland-Perry and Stallings, 1993; Krause and Oetzel, 2005). Body weight was measured (Ohaus digital scale, model CW-11, Newark, NJ) and BCS was assigned in quarter-unit increments for each cow weekly (Ferguson et al., 1994). More than one person assigned a BCS score independently at each time of scoring and the average score was used for statistical analysis.

Cow activity was monitored using the HOBO pendant G logger (Hobo Pendant G Acceleration Data Logger, Onset Computer Corp.) attached laterally to the distal left hind leg. The activity logger was attached to the leg using vet wrap. Data points were set to record at 60-s intervals. Data collected were used to calculate total lying time, bouts (number times laid down/24h), and duration of each lying bout (time from laying down to standing up), as well as standing time, standing bouts (number times stood/24h), and duration of each standing bout (time from standing up to laying down).

Calculations

Aflatoxin M1 (AFM1) excretion, was calculated as described by Maki et al. (2016a):

\[
\text{Excretion (µg/d)} = \text{concentration of AFM1 in milk on d 18 (µg/kg) } \times \text{ milk yield on d 18 (kg)}
\]

AF Transfer (%) = \[\text{AF excretion (µg/d)} / \text{ AFB1 intake (µg/d)}\] \times 100.

Statistical Analyses

The data were analyzed using the mixed model procedure of SAS (v 9.3; SAS Institute Inc., Cary, NC) to account for carryover effect by the following model
\[ y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + C_m + e_{ijklm}, \]

where \( y_{ijklm} \) is the observations for dependent variables; \( \mu \) is the general mean; \( S_i \) is the fixed effect of the \( i^{th} \) treatment sequence; \( A_{(i)j} \) is the random effect of the \( j^{th} \) cow in the \( i^{th} \) sequence; \( P_{(i)k} \) is the fixed effect of the \( k^{th} \) period; \( T_l \) is the fixed effect of the \( l^{th} \) treatment; \( C_m \) is the fixed carryover effect from the previous period (\( C = 0 \), if period = 1); \( e_{ijklm} \) is the random error. If carryover effects were not detected, data were analyzed as a replicated Latin square by the following model

\[ y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + D_m + T \times D_{lm} + e_{ijklm}, \]

where \( y_{ijklm} \) is the observation for dependent variables; \( \mu \) is the general mean. \( S_i \) is the random effect of the \( i^{th} \) square; \( A_{(i)j} \) is the random effect of the \( j^{th} \) cow in the \( i^{th} \) square; \( P_{(i)k} \) is the fixed effect of the \( k^{th} \) period; \( T_l \) is the fixed effect of the \( l^{th} \) treatment; \( D_m \) is the fixed effect of repeated measurement, which used as day in aflatoxin concentration and blood metabolites. The \( T \times D_{lm} \) term is the interaction of treatment and repeated measurement, and the interaction was removed if \( P > 0.3 \); \( e_{ijklm} \) is the random error. The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers (Littell et al., 2002). Variables were subjected to 5 covariance structures: compound symmetry, autoregressive order 1, autoregressive heterogeneous order 1, unstructured, and toeplitz. The covariance structure that yielded the lowest corrected Akaike information criterion was compound symmetry and used in the model (Littell et al., 2002).
A log10 transformation was used for the variable milk SCC for better homogeneity of the distribution of residuals. Means shown for this variable were back-transformed. Orthogonal contrasts were tested using the CONTRAST statement of SAS: CONT1 = POS (0% clay) compared with C; CONT2 = POS (0% clay) compared with the average of the three treatments (0.5%, 1%, or 2% clay) and linear and quadratic effects of treatments POS (0%), 0.5%, 1%, or 2% clay. Values reported are least squares means and associated standard errors of the mean. Residual distribution was evaluated for normality and homoscedasticity. A multivariable logistic mixed model (FREQ procedure) was used for the dichotomized variables (FS, GA, temperature, respiration, and heart rate). The chi-square was computed and is presented. Statistical significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

**RESULTS**

*Diet Composition*

The ingredient composition of the diet is in Table 3.1. Analyzed nutrients from the experimental diet are in Table 3.2. The physical characteristics of the TMR, based on the Penn State Particle Separator (Kononoff et al., 2003) were (mean ± SD) 5.1 ± 1% on upper sieve, 41.3 ± 3% on middle sieve, 40.7 ± 3% on lower sieve, and 12.9 ± 2% in the pan. Carryover effects were tested and were not present for any variable of interest ($P > 0.47$).

*DMI, BW, BCS, and Lactation Performance*

Performance data are in Table 3.3. There were no treatment differences for either contrasts (CONT1 or CONT2) for DMI, Milk Yield, BW, or BCS. Milk yield had a negative linear response as clay concentration increased ($P = 0.02$). Dry matter intake had a quadratic treatments effect ($P$
Fat-corrected milk (3.5%) tended to be higher for cows in POS compared with those receiving clay ($P = 0.06$; CONT2). Cows in POS had decreased feed conversion [3.5% FCM / DMI (kilogram / kilogram), $P = 0.02$; ECM / DMI (kilogram / kilogram), $P = 0.01$; and Milk yield / DMI (kilogram / kilogram), $P = 0.04$; CONT2]. Lactose yield (kilogram / d), and lactose concentration had a negative linear treatment effect ($P = 0.03$ and $P = 0.02$, respectively). Cows in POS tended to have more fat yield (kilogram / d) compared with cows receiving clay ($P = 0.09$; CONT2). The SNP tests had a negative linear treatment effect with the number of positive tests decreasing as the concentration of clay in the diet increased ($P = 0.004$). Total milk discarded (landfill due to regulations by the FDA on AFM$_1$ concentrations) was higher for cows in POS than milk from cows in C ($P < 0.0001$; CONT1).

**Aflatoxin Concentrations**

Aflatoxin concentrations in TMR were below detection limits. Aflatoxin concentrations in milk, urine, feces, and rumen fluid are in Table 4 and Figures 3.1, 3.2, and 3.3. Milk AFM$_1$ concentrations were lower ($P < 0.0001$) for cows receiving clay than cows that were not receiving clay (POS). Cows in C had lower ($P = 0.004$) urine AFM$_1$ concentrations than cows in POS (CONT1). Fecal and rumen fluid samples from cows receiving clay had lower concentrations of AFB$_1$ than cows not receiving clay ($P = 0.01$ and $P = 0.004$, respectively; CONT2). There was a day effect for milk, feces, and rumen samples ($P < 0.0001$); and a treatment by day effect (feces, $P = 0.0031$; rumen fluid and milk, $P < 0.0001$).

**Serum and Plasma Chemistry Profile**

Serum chemistry profiles are in Table 3.5. Cows in POS tented to have increased blood total protein concentrations ($P = 0.08$), and had greater blood concentrations for globulin ($P = 0.02$), GGT ($P = 0.07$) than cows in C (CONT1). Cows in POS had lower blood concentrations
for albumin:globulin ratio \((P = 0.04)\), AST \((P = 0.03)\), cholesterol \((P = 0.005)\), and GLDH \((P = 0.04)\) than cows in C (CONT1). Cows in POS had higher CPK serum concentrations compared with cows receiving clay \((P = 0.04;\text{ CONT2})\). Serum concentrations for alkaline phosphate \((P = 0.07)\) and cholesterol \((P = 0.06)\) tended to have a positive linear treatment effect, whereas a negative linear clay treatment effect was observed for GGT \((P = 0.05)\), and a positive clay treatment effect for CPK \((P = 0.02)\).

Cows in POS tended \((P = 0.06)\) to have higher plasma SOD concentrations than cows in C (CONT1). Cows in POS tended to have higher plasma SOD concentrations than cows receiving clay \((P = 0.07;\text{ CONT2})\). There tended \((P = 0.06)\) to be a quadratic treatment effect for plasm SOD concentrations. There were no treatment differences for plasma vitamins A, D, and E concentrations (Table 3.6).

**Health and Activity**

There was no difference among treatments for all health parameters measured \((P > 0.20)\). Cows receiving clay spent more time standing than cows not receiving clay \((\text{POS} = 701.4\text{ min and C} = 726.7\text{ min};\text{ }P = 0.05;\text{ CONT2})\). There was a positive linear treatment effect for the total standing time \((0.5\% = 730.3\text{ min, }1\% = 739.9\text{ min, and }2\% = 761.4\text{ min, }P = 0.03)\) and the average duration of standing behavior \((0.5\% = 77.5\text{ min, }1\% = 83.5\text{ min, }2\% = 91.0\text{ min, }P = 0.05)\).

**DISCUSSION**

The aims of this study were to determine the effects of a commercially available clay product in response to an aflatoxin \((\text{AFB}_1)\) challenge on AF excretion, blood chemistry, immune
response, milk composition and health of mid-lactation Holstein cows; and to determine the most appropriate clay concentration to be used. We postulated that the use of clay would lower AF excretion rate while maintaining health status and lactation performance through an AF challenge.

Clay feed additives have been shown to decrease AF excretion and AF transfer from feed to milk (Kutz et al., 2009; Kissell et al., 2013; Barrientos-Velzaquez et al., 2016; Maki et al., 2016a). Some studies have reported no changes in DMI or milk yield when feeding clay products during an AF challenge (Battacone et al., 2009; Queiroz et al., 2012; Maki et al., 2016a; Maki et al., 2016b). However, in the current study, there was a quadratic treatment effect for DMI and a negative linear treatment effect for milk yield. The small changes in these values caused significant differences in 3.5% FCM, 3.5% FCM / DMI, ECM / DMI, and Milk / DMI. Perhaps the differences seen in milk yield that reflect negatively on efficiency parameters could be the result of the cow’s metabolism of AF. Kubena et al. (1998) reported a reduction in feed consumption that adversely affected feed conversion by broiler chickens exposed to AF.

In the present study, cows fed the clay-based product had a lower number of positive SNP tests and AF transfer from the rumen (challenge) to milk. As clay increased, AFM1 concentration decreased and the highest reduction occurred in cows receiving 2%. Queiroz et al. (2012) reported an increase in AF excretion in milk at low concentrations of dietary clay inclusion (0.2% of dietary DM) but when clay was increased to 1% of dietary DM there was a 16% decrease in AF excretion. Maki et al. (2016a) used a clay feed additive at 0.5% and 1% of dietary DM and found both percentages decreased AFM1 concentration in milk (51.3% and 69.7%, respectively). In the present study, there was a significant decrease in AFM1 excretion
(µg/d) that resulted in a reduction of 25% (0.5%), 18% (1%) and 41% (2%), which was seen as a decrease in the AF transfer percentage.

Few data have been reported on bodily excretions of AF. However, fecal concentrations of AFB₁ was measured by Hoogenboom et al. (2001). To do this, two types of aflatoxin had been fed to cows, one that was pure AFB₁ or known as unaltered and an AFB₁ that had been labeled with a radioactive agent that underwent decontamination so as to not cause harm to the animal and was intended to have reduced bioavailability to the animal so it would in fact be excreted through the digestive system. The purpose was to determine AF absorption and excretion within body tissues. For feces, almost all of the radiolabeled AFB₁ had been excreted in the feces which resulted in lower concentrations of AF found in milk and tissues. A smaller portion of the original untreated AFB₁ was excreted in the feces and greater concentrations of AF were found in milk and tissues. In the present study, fecal concentrations of AFB₁ decreased linearly with clay increasing in the diet. Since clay products have such a high affinity for aflatoxins, it is plausible that the clay used in the present study had a high affinity for AF and thus was not able to be detected as pure unaltered AFB₁. In vitro experiments suggest that clays have a sequestering capacity for aflatoxins anywhere from 80 to 100% of aflatoxin present; however, pH, proteins, and inorganic nutrients have been reported to play a role in decreasing the sequestering capacity of AF (Lemke et al., 2001; Moschini et al., 2008; Trckova et al., 2014; Barrientos-Velazques et al., 2016). Barrientos-Velazques et al. (2016) reported that when artificial gastric fluid was introduced to an in vitro system, the adsorption of AF was reduced by 60% using bentonite. The normal mechanism for adsorbents to bind with AF is by sequestration of AF in the gastrointestinal tract and chemisorption, tight bonding between the molecules
(Kubena et al., 1998; Hoogenboom et al., 2001). This may explain why the rumen concentration of AF and fecal concentrations were significantly decreased for cows fed clay in the diet.

Urine AF concentrations have been very well documented for humans and have been used as a short-term biomarker of AFB\textsubscript{1} exposure (Wang et al., 2008). We are not aware of any studies that reported AFM\textsubscript{1} presence in urine of dairy cows. Differences in AF concentrations in urine between POS and C suggest that cows are excreting the toxin through many other physiological systems (i.e., urinary) and not just through the digestive system and mammary gland. This supports the theory that when the liver goes through the activation pathways described by Hsieh and Atkinson (1991) the hydroxylated AFM\textsubscript{1} is spread through the body where different systems such as excretory and mammary gland are exposed and AF is thus excreted in the urine (Hsieh and Atkinson, 1991; Eaton and Gallagher, 1994; Jager et al., 2016). Kidneys are larger in chickens that are exposed to AF than not exposed to AF (Kubena et al., 1998; Fowler et al., 2015). This phenomenon is still to be determined in situations where the AF challenge to the cow is not as intense (i.e., contaminated feed) as the one imposed in the present study.

Even though clays have been reported to decrease AF, certain vitamins (A, D, and E) and minerals have been decreased in the presence of smectite clays (Tang et al., 2009; Barrientos-Velazques et al., 2016). In the present study, there were no significant differences among treatment groups suggesting that AF was not altering vitamin and mineral concentrations as previously 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 present study, Maki et al. (2016b) found no interference with serum Vitamin A concentrations when montmorillonite clay was fed to bovine animals at 18 and 20 kg/d. Witzemann (1985) showed that CPK played a role in ion
transport across membranes in multiple processes such as glycolysis, muscle contractions, ATP-dependent releases of neurotransmitters and others. With this implication, data from the present study could indicate that CPK is involved in aiding ion transport of AF as clay increased in the diet. In contrast, AST and GLDH were not affected by clay treatment, but with the introduction of AF to the diet, their concentrations significantly decreased. Stojević et al. (2005) showed a normal range of AST to be between 19 and 84 U/L. In the present study, cows in C were on the higher end of the aforementioned interval for AST at 84 U/L. Cows introduced to AF decreased AST by 5 U/L. There tended to be a quadratic treatment effect that allowed cows being fed 2% clay to increase AST to 85 U/L, which is comparable to the reported normal concentration by cows in C. However, changes in AST have been reported to be associated with disease and indicative of liver condition (Mohammad et al., 2011; Randhawa et al., 2014). Similarly, cows in C had GLDH serum concentrations of 91 U/L but when cows were introduced to AF (POS) the serum concentrations of GLDH decreased to 75 U/L. At 1% clay, the trend continued and GLDH returned to normal concentrations at 81 U/L. Schulz et al. (2014) reported an increase in serum GLDH for cows with higher BCS postpartum. Additionally, in cows experiencing high BCS, the risk of metabolic disorders, including fatty liver disease, were higher as well than cows with low GLDH. However, no differences in BCS were reported. The short length of each period combined with the fact that cows in this study were at mid-lactation; show that there would not be sufficient time to see significant changes in BCS. Along with AST, GLDH is another indicator of liver functionality and thus, decreased values in the presence of AF could indicate alterations, possibly suppression, in liver functionality. Similar results in broiler chicks were found by Kubena et al. (1998) when there were reduced serum concentrations of cholesterol which agrees with the cholesterol concentrations found in the present study. With the presence of
clay, the liver appears to regain its normal function by means of cholesterol concentrations similar to the cows in C. Trckova et al. (2014) tested similar blood chemistry parameters in pigs supplemented with bentonite and reported a 11 U/L decrease in serum AST, at 20 g/kg (2% bentonite) inclusion, which could support this theory; however, more research is needed to understand the altered liver functionality in the presence of AF in dairy cows.

The role of SOD is related to oxidative stress and maintaining normal cellular function in cattle (Bernabucci et al., 2002; Yuan et al., 2012). Perhaps, as AF was introduced as a challenge, cows underwent a greater degree of oxidative stress according to the tendency for increased serum SOD concentrations in cows in POS than C. Machado et al. (2014), reported that serum SOD activity for cows treated with injectable trace minerals was greater than control (16 and 13 U/mL, respectively). Superoxide dismutases are enzymes that are involved in the anti-oxidant system and are Mn⁺, Cu⁺ and Zn⁺ dependent (Machado et al., 2014). Additionally, they reported that serum SOD activity was 13 and 15 U/mL for cows diagnosed with mastitis and unaffected, respectively. Therefore, it seems that higher concentrations of SOD in serum or plasma may be correlated to the higher levels of aflatoxin preventing the liver from oxidative stress. However, Xiong et al. (2015) reported a decrease in SOD in the presence of AF. In that experiment, cows were challenged with lower concentrations of AFB₁ (20 µg/kg or 40 µg/kg of dietary DMI) in the TMR than our present study where cows were directly challenged through the rumen-cannula with (100 µg/kg of dietary DMI).

Ogunade et al. (2016) studied the effects of adding 3 mycotoxin-sequestering agents (SEQ) to diets contaminated with AFB₁ (75 µg/kg of dietary DMI) on reducing milk aflatoxin M₁ and immune status of dairy cows. The authors reported that the greater mean fluorescent intensity of staining for CD62L and CD18 on neutrophils 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 SOD plasma concentrations possibly meaning less oxidative stress. Additional studies that report blood chemistry analysis while feeding clay-based products are needed for a better understanding of the association of AF and oxidative stress in dairy cows.

CONCLUSIONS

The inclusion of this clay product, closely resembling the structure of vermiculite, illite, or montmorillonite, linearly reduced aflatoxin transfer from the rumen (challenge) to the milk and feces of mid-lactation Holstein cows. Our results seem to indicate that this clay product does not improve or aid the cow’s efficiency or health status when being challenged with AF. Although the clay treatment groups did not maintain efficiency or milk yield (at 2% inclusion), AF concentrations in rumen, milk, and feces were decreased and there was no reduction in absorption for the analyzed vitamins. Increased serum concentrations of CPK and the decreased concentrations of AST and GLDH in challenged cows not fed clay compared with cows challenged and receiving clay indicated improved liver function for the latter.
### Tables and Figures

**Table 3.1** Ingredient composition of the lactation diet fed to all cows throughout the experimental period.

<table>
<thead>
<tr>
<th>Ingredient,</th>
<th>% of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa hay</td>
<td>8.20</td>
</tr>
<tr>
<td>Corn silage</td>
<td>32.8</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>7.20</td>
</tr>
<tr>
<td>Wet brewers grains</td>
<td>7.49</td>
</tr>
<tr>
<td>Dry ground corn grain</td>
<td>21.55</td>
</tr>
<tr>
<td>Soy hulls</td>
<td>10.42</td>
</tr>
<tr>
<td>Soybean meal, 48%</td>
<td>3.25</td>
</tr>
<tr>
<td>Expeller soybean meal¹</td>
<td>3.30</td>
</tr>
<tr>
<td>Molasses, sugarbeet</td>
<td>2.05</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.13</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.35</td>
</tr>
<tr>
<td>Bypass fat²</td>
<td>1.87</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.34</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.81</td>
</tr>
<tr>
<td>Salt (plain)</td>
<td>0.04</td>
</tr>
<tr>
<td>Mineral and vitamin mix³</td>
<td>0.20</td>
</tr>
</tbody>
</table>

¹ SoyPlus® (West Central, Ralston, IA)
² Energy Booster 100® (Milk Specialties Global, Paris, IL)
³ Mineral and Vitamin mix was formulated with 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5,000 mg/kg of Cu, 250 mg/kg of I, 40 mg/kg of Co, 150 mg/kg of Se, 2,200 kIU/kg of vitamin A, 660 kIU/kg of vitamin D₃, and 7,700 IU/kg of vitamin E.
Table 3.2 Mean chemical composition and associated standard deviations for diets fed to all cows throughout the experimental period.

<table>
<thead>
<tr>
<th>Item</th>
<th>Period¹</th>
<th></th>
<th></th>
<th></th>
<th>SD²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>48.4</td>
<td>44.5</td>
<td>47.3</td>
<td>46.4</td>
<td>43.1</td>
</tr>
<tr>
<td>CP, % of DM</td>
<td>15.8</td>
<td>14.5</td>
<td>15.1</td>
<td>14.8</td>
<td>14.6</td>
</tr>
<tr>
<td>ADF, % of DM</td>
<td>22.5</td>
<td>22.0</td>
<td>22.2</td>
<td>22.2</td>
<td>24.2</td>
</tr>
<tr>
<td>NDF, % of DM</td>
<td>32.4</td>
<td>32.7</td>
<td>32.6</td>
<td>34.1</td>
<td>31.6</td>
</tr>
<tr>
<td>Lignin, % of DM</td>
<td>4.0</td>
<td>3.3</td>
<td>4.1</td>
<td>3.4</td>
<td>3.9</td>
</tr>
<tr>
<td>Starch, % of DM</td>
<td>27.4</td>
<td>29.9</td>
<td>28.8</td>
<td>28.9</td>
<td>27.4</td>
</tr>
<tr>
<td>Crude fat, % of DM</td>
<td>5.5</td>
<td>4.9</td>
<td>5.2</td>
<td>4.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Ash, % of DM</td>
<td>6.54</td>
<td>6.89</td>
<td>6.06</td>
<td>6.38</td>
<td>7.06</td>
</tr>
<tr>
<td>TDN, % of DM³</td>
<td>72</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>71</td>
</tr>
<tr>
<td>NE₁, Mcal/kg of DM</td>
<td>1.71</td>
<td>1.71</td>
<td>1.74</td>
<td>1.74</td>
<td>1.66</td>
</tr>
<tr>
<td>Ca, % of DM</td>
<td>0.71</td>
<td>0.76</td>
<td>0.64</td>
<td>0.66</td>
<td>0.69</td>
</tr>
<tr>
<td>P, % of DM</td>
<td>0.40</td>
<td>0.30</td>
<td>0.31</td>
<td>0.31</td>
<td>0.35</td>
</tr>
<tr>
<td>Mg, % of DM</td>
<td>0.22</td>
<td>0.20</td>
<td>0.19</td>
<td>0.20</td>
<td>0.26</td>
</tr>
<tr>
<td>K, % of DM</td>
<td>1.29</td>
<td>1.16</td>
<td>1.07</td>
<td>1.02</td>
<td>1.41</td>
</tr>
<tr>
<td>Na, % of DM</td>
<td>0.23</td>
<td>0.26</td>
<td>0.23</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>S, % of DM</td>
<td>0.22</td>
<td>0.19</td>
<td>0.19</td>
<td>0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>Fe, ppm</td>
<td>711</td>
<td>699</td>
<td>522</td>
<td>631</td>
<td>674</td>
</tr>
<tr>
<td>Zn, ppm</td>
<td>94</td>
<td>89</td>
<td>84</td>
<td>93</td>
<td>85</td>
</tr>
<tr>
<td>Cu, ppm</td>
<td>17</td>
<td>17</td>
<td>16</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Mn, ppm</td>
<td>90</td>
<td>96</td>
<td>77</td>
<td>86</td>
<td>87</td>
</tr>
<tr>
<td>Mo, ppm</td>
<td>0.7</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

¹ Period: made up of 3 consecutive weeks. Cows were fed the TMR ad libitum. Each cow was provided with a mixture of 500 g of ground corn and clay at different percentages; 0, 0.5, 1, or 2% of dietary DM. The clay was split into two top dress and fed immediately after feeding and again after 14 h.
² Maximum within period SD.
³ NRC (2001).
Table 3.3 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

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>POS</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>C</th>
<th>SEM</th>
<th>Contrasts²</th>
<th>Linear</th>
<th>Quad</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d</td>
<td>21.54</td>
<td>21.81</td>
<td>22.34</td>
<td>21.43</td>
<td>21.58</td>
<td>0.69</td>
<td>0.94</td>
<td>0.40</td>
<td>0.79</td>
</tr>
<tr>
<td>BW, kg</td>
<td>669.8</td>
<td>665.7</td>
<td>675.1</td>
<td>669.0</td>
<td>667.6</td>
<td>20.5</td>
<td>0.58</td>
<td>0.97</td>
<td>0.75</td>
</tr>
<tr>
<td>BCS</td>
<td>3.17</td>
<td>3.60</td>
<td>3.13</td>
<td>3.09</td>
<td>2.86</td>
<td>0.28</td>
<td>0.43</td>
<td>0.74</td>
<td>0.53</td>
</tr>
<tr>
<td>Milk yield</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield kg/d</td>
<td>37.83</td>
<td>37.57</td>
<td>37.28</td>
<td>36.44</td>
<td>38.57</td>
<td>1.49</td>
<td>0.24</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>3.5% FCM</td>
<td>41.37</td>
<td>38.22</td>
<td>39.32</td>
<td>38.40</td>
<td>42.85</td>
<td>1.81</td>
<td>0.42</td>
<td>0.06</td>
<td>0.20</td>
</tr>
<tr>
<td>ECM</td>
<td>39.62</td>
<td>37.10</td>
<td>37.10</td>
<td>37.11</td>
<td>40.90</td>
<td>1.60</td>
<td>0.38</td>
<td>0.05</td>
<td>0.16</td>
</tr>
<tr>
<td>AFM; Snap³</td>
<td>14.2</td>
<td>13.9</td>
<td>13.1</td>
<td>12.4</td>
<td>0</td>
<td>0.44</td>
<td>&lt;0.0001</td>
<td>0.04</td>
<td>0.004</td>
</tr>
<tr>
<td>Milk discarded, kg/d⁴</td>
<td>255.0</td>
<td>256.1</td>
<td>261.6</td>
<td>253.1</td>
<td>0</td>
<td>7.05</td>
<td>&lt;0.0001</td>
<td>0.79</td>
<td>0.92</td>
</tr>
<tr>
<td>Milk composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat, %</td>
<td>4.12</td>
<td>3.68</td>
<td>3.84</td>
<td>3.86</td>
<td>4.19</td>
<td>0.22</td>
<td>0.76</td>
<td>0.15</td>
<td>0.60</td>
</tr>
<tr>
<td>Fat, kg/d</td>
<td>1.54</td>
<td>1.36</td>
<td>1.43</td>
<td>1.40</td>
<td>1.60</td>
<td>0.09</td>
<td>0.54</td>
<td>0.09</td>
<td>0.32</td>
</tr>
<tr>
<td>Protein, %</td>
<td>2.86</td>
<td>2.87</td>
<td>2.90</td>
<td>2.88</td>
<td>2.81</td>
<td>0.05</td>
<td>0.15</td>
<td>0.48</td>
<td>0.50</td>
</tr>
<tr>
<td>Protein, kg/d</td>
<td>1.08</td>
<td>1.07</td>
<td>1.07</td>
<td>1.05</td>
<td>1.07</td>
<td>0.04</td>
<td>0.93</td>
<td>0.49</td>
<td>0.24</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>4.72</td>
<td>4.70</td>
<td>4.70</td>
<td>4.64</td>
<td>4.66</td>
<td>0.04</td>
<td>0.11</td>
<td>0.17</td>
<td>0.03</td>
</tr>
<tr>
<td>Lactose, kg/d</td>
<td>1.78</td>
<td>1.75</td>
<td>1.75</td>
<td>1.69</td>
<td>1.78</td>
<td>0.07</td>
<td>0.99</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>MUN, mg/dL</td>
<td>10.88</td>
<td>10.54</td>
<td>10.67</td>
<td>10.85</td>
<td>10.38</td>
<td>0.42</td>
<td>0.36</td>
<td>0.65</td>
<td>0.91</td>
</tr>
<tr>
<td>SCC, log transformed</td>
<td>4.85</td>
<td>4.85</td>
<td>4.75</td>
<td>4.85</td>
<td>4.57</td>
<td>0.34</td>
<td>0.21</td>
<td>0.85</td>
<td>0.95</td>
</tr>
<tr>
<td>3.5% FCM/DMI, kg/kg</td>
<td>1.95</td>
<td>1.77</td>
<td>1.75</td>
<td>1.80</td>
<td>1.97</td>
<td>0.09</td>
<td>0.81</td>
<td>0.02</td>
<td>0.22</td>
</tr>
<tr>
<td>ECM/DMI, kg/kg</td>
<td>1.86</td>
<td>1.72</td>
<td>1.69</td>
<td>1.74</td>
<td>1.88</td>
<td>0.07</td>
<td>0.83</td>
<td>0.01</td>
<td>0.19</td>
</tr>
<tr>
<td>Milk/DMI, kg/kg</td>
<td>1.76</td>
<td>1.73</td>
<td>1.67</td>
<td>1.71</td>
<td>1.78</td>
<td>0.06</td>
<td>0.78</td>
<td>0.04</td>
<td>0.14</td>
</tr>
</tbody>
</table>

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 Number of milkings with a positive snap test.

⁴ Total amount of milk discarded during the challenge week.
Table 3.4 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

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>POS</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>C</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Contrasts²</td>
</tr>
<tr>
<td>Milk, AFM₁ (µg/kg)³</td>
<td>0.43</td>
<td>0.35</td>
<td>0.30</td>
<td>0.25</td>
<td>0.00</td>
<td>0.06</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Milk, AFM₁ d 18 (µg/kg)</td>
<td>0.80</td>
<td>0.58</td>
<td>0.58</td>
<td>0.47</td>
<td>0.00</td>
<td>0.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AFM Excretion, (µg/d)⁴</td>
<td>27.81</td>
<td>20.83</td>
<td>22.82</td>
<td>16.51</td>
<td>0.00</td>
<td>3.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AFM Transfer, (%)⁵</td>
<td>1.37</td>
<td>1.01</td>
<td>0.98</td>
<td>0.74</td>
<td>0.00</td>
<td>0.16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urine, AFM₁ (µg/kg)⁶</td>
<td>6.50</td>
<td>8.60</td>
<td>4.38</td>
<td>5.51</td>
<td>0.01</td>
<td>1.37</td>
<td>0.004</td>
</tr>
<tr>
<td>Feces, AFB₁ (µg/kg)³</td>
<td>2.78</td>
<td>1.79</td>
<td>1.52</td>
<td>1.48</td>
<td>0.16</td>
<td>0.35</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Rumen fluid, AFB₁ (µg/kg)⁷</td>
<td>0.10</td>
<td>0.05</td>
<td>0.02</td>
<td>0.02</td>
<td>0.003</td>
<td>0.03</td>
<td>0.001</td>
</tr>
</tbody>
</table>

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 AFM₁. POS = 35.62 kg, 0.5% = 35.58 kg, 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 14, 18, and 21 of each period.

⁷ Samples that were analyzed were collected on d 18 of each period.

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Table 3.5 Least squares means and associated SEM for serum metabolites harvested daily from d 15 to 21 (challenge period) in 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 (C) treatments

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Treatment¹</th>
<th>P-value</th>
<th>Contrasts²</th>
<th>Linear</th>
<th>Quad Trt</th>
<th>Trt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POS</td>
<td>0.5%</td>
<td>1%</td>
<td>2%</td>
<td>C</td>
<td>SEM</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.791</td>
<td>0.794</td>
<td>0.788</td>
<td>0.778</td>
<td>0.776</td>
<td>0.02</td>
</tr>
<tr>
<td>BUN (Urea), mg/dL</td>
<td>9.30</td>
<td>9.50</td>
<td>9.73</td>
<td>9.68</td>
<td>9.21</td>
<td>0.30</td>
</tr>
<tr>
<td>Total protein, g/dL</td>
<td>7.36</td>
<td>7.36</td>
<td>7.31</td>
<td>7.39</td>
<td>7.27</td>
<td>0.08</td>
</tr>
<tr>
<td>Albumin, g/dL</td>
<td>3.26</td>
<td>3.21</td>
<td>3.24</td>
<td>3.24</td>
<td>3.25</td>
<td>0.03</td>
</tr>
<tr>
<td>Globulin, g/dL</td>
<td>4.10</td>
<td>4.15</td>
<td>4.07</td>
<td>4.15</td>
<td>4.02</td>
<td>0.08</td>
</tr>
<tr>
<td>Albumin/Globulin ratio</td>
<td>0.796</td>
<td>0.783</td>
<td>0.811</td>
<td>0.792</td>
<td>0.819</td>
<td>0.02</td>
</tr>
<tr>
<td>Calcium, mg/dL</td>
<td>9.32</td>
<td>9.36</td>
<td>9.27</td>
<td>9.27</td>
<td>9.41</td>
<td>0.06</td>
</tr>
<tr>
<td>Phosphorus, mg/dL</td>
<td>5.54</td>
<td>5.22</td>
<td>5.43</td>
<td>5.39</td>
<td>5.49</td>
<td>0.16</td>
</tr>
<tr>
<td>Sodium, mmol/L</td>
<td>137.2</td>
<td>136.9</td>
<td>137.0</td>
<td>136.9</td>
<td>137.6</td>
<td>0.31</td>
</tr>
<tr>
<td>Potassium, mmol/L</td>
<td>4.21</td>
<td>4.33</td>
<td>4.21</td>
<td>4.23</td>
<td>4.33</td>
<td>0.08</td>
</tr>
<tr>
<td>Na:K Ratio</td>
<td>32.81</td>
<td>32.14</td>
<td>32.79</td>
<td>32.72</td>
<td>32.12</td>
<td>0.6</td>
</tr>
<tr>
<td>Chloride, mmol/L</td>
<td>95.22</td>
<td>95.89</td>
<td>95.59</td>
<td>94.95</td>
<td>95.84</td>
<td>0.40</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>63.40</td>
<td>63.39</td>
<td>63.05</td>
<td>64.36</td>
<td>64.18</td>
<td>0.79</td>
</tr>
<tr>
<td>Alkaline phosphate total, U/L</td>
<td>46.97</td>
<td>45.19</td>
<td>49.43</td>
<td>49.96</td>
<td>46.52</td>
<td>1.43</td>
</tr>
<tr>
<td>AST³, U/L</td>
<td>79.17</td>
<td>74.21</td>
<td>84.69</td>
<td>77.01</td>
<td>84.23</td>
<td>5.88</td>
</tr>
<tr>
<td>GGT⁴, U/L</td>
<td>30.97</td>
<td>31.97</td>
<td>31.25</td>
<td>30.25</td>
<td>29.92</td>
<td>1.63</td>
</tr>
<tr>
<td>Total bilirubin, mg/dL</td>
<td>0.19</td>
<td>0.19</td>
<td>0.20</td>
<td>0.19</td>
<td>0.19</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 3.5 continued

<table>
<thead>
<tr>
<th>Test</th>
<th>126.3</th>
<th>131.0</th>
<th>145.5</th>
<th>143.8</th>
<th>133.7</th>
<th>9.30</th>
<th>0.37</th>
<th>0.04</th>
<th>0.02</th>
<th>0.23</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK, U/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol total, mg/dL</td>
<td>227.4</td>
<td>228.1</td>
<td>235.2</td>
<td>233.3</td>
<td>238.6</td>
<td>8.3</td>
<td>0.005</td>
<td>0.12</td>
<td>0.06</td>
<td>0.30</td>
</tr>
<tr>
<td>GLDH, U/L</td>
<td>75.81</td>
<td>62.37</td>
<td>81.56</td>
<td>69.04</td>
<td>91.02</td>
<td>26.4</td>
<td>0.04</td>
<td>0.44</td>
<td>0.81</td>
<td>0.70</td>
</tr>
<tr>
<td>Bicarbonate (TCO2), mmol/L</td>
<td>30.12</td>
<td>29.88</td>
<td>29.88</td>
<td>30.31</td>
<td>29.85</td>
<td>0.43</td>
<td>0.54</td>
<td>0.79</td>
<td>0.56</td>
<td>0.35</td>
</tr>
<tr>
<td>Magnesium, mg/dL</td>
<td>2.28</td>
<td>2.23</td>
<td>2.27</td>
<td>2.25</td>
<td>2.25</td>
<td>0.04</td>
<td>0.46</td>
<td>0.35</td>
<td>0.80</td>
<td>0.69</td>
</tr>
<tr>
<td>Anion gap, mEq/L</td>
<td>16.10</td>
<td>15.94</td>
<td>15.70</td>
<td>15.77</td>
<td>16.32</td>
<td>0.54</td>
<td>0.34</td>
<td>0.38</td>
<td>0.51</td>
<td>0.46</td>
</tr>
<tr>
<td>SOD, U/mL</td>
<td>2.72</td>
<td>2.16</td>
<td>1.90</td>
<td>2.30</td>
<td>1.96</td>
<td>0.30</td>
<td>0.06</td>
<td>0.07</td>
<td>0.38</td>
<td>0.06</td>
</tr>
<tr>
<td>GSH, µM</td>
<td>8.56</td>
<td>7.27</td>
<td>8.34</td>
<td>8.73</td>
<td>10.7</td>
<td>3.7</td>
<td>0.68</td>
<td>0.92</td>
<td>0.90</td>
<td>0.86</td>
</tr>
</tbody>
</table>

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%. Day differed (P < 0.05) for total protein, globulin, sodium, sodium:potassium ratio, CPK, TCO2, magnesium, and anion gap. TRT × DAY interaction was not present (P > 0.15) for all variables.

3 Aspartate aminotransferase.

4 Gamma-glutamyl transpeptidase.

5 Creatine phosphokinase.

6 Glutamate dehydrogenase.

7 Superoxide dismutase. One unit (U) is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

8 Glutathione.
Table 3.6 Least squares means and associated SEM for serum vitamin profiles 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

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>Treatment¹</th>
<th>P-value</th>
<th>SEM</th>
<th>Contrasts²</th>
<th>Linear</th>
<th>Quad</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POS</td>
<td>0.5%</td>
<td>1%</td>
<td>2%</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Vitamin A, ng/mL³</td>
<td>260.8</td>
<td>261.0</td>
<td>287.6</td>
<td>269.9</td>
<td>267.1</td>
<td>23.21</td>
</tr>
<tr>
<td>Vitamin D, nmol/L</td>
<td>137.0</td>
<td>155.8</td>
<td>143.4</td>
<td>133.3</td>
<td>153.1</td>
<td>7.81</td>
</tr>
<tr>
<td>Vitamin E, µg/mL</td>
<td>4.99</td>
<td>5.06</td>
<td>4.81</td>
<td>4.96</td>
<td>4.72</td>
<td>0.33</td>
</tr>
<tr>
<td>Vit E : Chol ratio (×10⁻³)</td>
<td>2.07</td>
<td>2.04</td>
<td>2.02</td>
<td>2.05</td>
<td>2.08</td>
<td>0.1</td>
</tr>
</tbody>
</table>

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 diet (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%.

³ Vitamin results are based on every period pre-aflatoxin challenge. Blood was draw on d 14 of each period.
Figure 3.1 Least squares means and associated SEM for milk concentrations of AFM$_1$ in response to an aflatoxin challenge (d 15 to 17) for cows in positive control with no clay (POS), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and a negative control (C) treatments from d 18 to 21 of each period. On d 18 AFM$_1$ concentrations in milk differed ($P < 0.0001$). TRT × DAY: $P < 0.0001$. Horizontal solid line represents the FDA allowable AFM$_1$ concentration in milk (0.5 µg/kg).
Figure 3.2 Least squares means and associated SEM for fecal concentrations of AFB$_1$ in response to an aflatoxin challenge (d 15 to 17) for cows in positive control with no clay (POS), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and a negative control (C) treatments from d 18 to 21 of each period. On d 18 ($P < 0.0001$) AFB$_1$ concentrations in feces differed. TRT × DAY: $P = 0.009$. 
Figure 3.3 Least squares means and associated SEM for rumen fluid concentrations of AFB\(_1\) in response to an aflatoxin challenge (d 15 to 17) for cows in positive control with no clay (POS), 0.5% clay (0.5%), 1% clay (1%), 2% clay (2%), and a negative control (C) treatments from d 18 to 21 of each period. On d 18 (\(P < 0.0001\)) AFB\(_1\) concentrations in rumen fluid differed. TRT \(\times\) DAY: \(P < 0.0001\).
REFERENCES


CHAPTER 4

Dietary clay supplementation improves hepatic expression of inflammatory markers in Holstein cows challenged with aflatoxin

ABSTRACT

Oral supplementation of clay to dairy cattle has been reported to reduce toxicity of aflatoxin (AF) in contaminated feed. The objective of this study was to determine the effects of 3 concentrations of dietary clay supplementation (EcoMix®) after an AF challenge on hepatic gene expression of seven different inflammation markers. Ten multiparous rumen-cannulated Holstein cows [BW (mean ± SD) = 669 ± 20 kg and 146 ± 69 DIM] were assigned to 1 of 5 treatments in a randomized replicated 5 × 5 Latin square design balanced to measure carryover effects. Periods (21 d) were divided into an adaptation phase (d 1 to 14) and a measurement phase (d 15 to 21). From d 15 to 17 cows received an AF challenge consisting of 100 μg of Aflatoxin B1 (AFB1)/kg of dietary DMI. AFB1 was fitted into 10-mL gelatin capsules (TORPAC, Fairfield, NJ) and administered into the rumen through the cannula based on the average DMI obtained on d 12 to 14. Treatments were: POS, no clay plus an AF challenge; three different concentrations of clay (0.5%, 1%, or 2% of dietary DMI) plus an AF challenge; and control (C), no clay and no AF challenge. Statistical analysis was performed using the MIXED procedure of SAS. Contrasts included CONT1 (POS vs. C), CONT2 (POS vs. the average of 0.5%, 1%, or 2%), and tests of linear and quadratic treatment effects of clay inclusion. When comparing POS with C, the AF challenge caused a 2.27-fold downregulation of HP (P = 0.04) and tended to have a 1.06-fold downregulation of STAT3 (P = 0.10). However, when supplemented with clay, cows had a linear
increased hepatic expression for *NFKB1* (*P* = 0.02) and a trend for linear increased for *TNFA* (*P* = 0.10). In conclusion, the gene expression profile suggested that an AF challenge suppressed liver inflammation markers and there was a restorative effect when orally supplemented with clay that seemed to counteract the immunosuppression of AF.

**Key words:** clay, aflatoxin, hepatic expression, inflammation.
INTRODUCTION

One of the most toxic secondary metabolites to ruminant animals is produced by fungi from the genus *Aspergillus* (Plasencia, 2005; Whitlow and Hagler, 2005; Gallo et al., 2015). *Aspergillus* species produce aflatoxins (AF) and are commonly found in the soil and on feedstuffs throughout the growing season, in storage, and after processing (Betran et al., 2005; Guo et al, 2005; Kabet and Var 2006). Among aflatoxins, B$_1$ (AFB$_1$) is the most toxic as, once ingested, it is metabolized by the liver into AFM$_1$, a hydroxyl compound that can be excreted in milk (Abrar et al., 2013; Barrientos-Velazquez et al., 2016). Since AF are hepatotoxic, they have been classified as a group 1 carcinogen (IARC, 2002), which becomes a major consumer safety issue.

In order to alleviate the adverse effects of AF, feeding a dietary clay supplement has been extensively studied and proven to alleviate the effects of AF on health. Wang et al. (2008) tested the efficacy of NovaSil clay in humans exposed to AF by delivering capsules before meals for three months. The study found that serum and urine AF biomarkers were significantly reduced when the calcium montmorillonite product was supplemented.

Countless data have been generated on the metabolism of AF, yet very limited research has been steered toward its genetic effects on liver markers in bovine. Earlier reports by Butler (1970) linked inflammatory responses with the hepatotoxic effects of AFB$_1$. More recent reports, by Hinton et al. (2016) tested intermittent and continuous AF dosing to rats for 40 wk and found evidence to suggest induction of an inflammatory response at 12 wk. Since AF are metabolized by the liver and because inflammation is the start of programed cell death processes (Yu et al., 2009; Hinton et al., 2016), the purpose of this study was to determine the effects of AF, as well as the effects of clay, during an AF challenge on known inflammation markers in bovine species.
MATERIALS AND METHODS

Animal care and housing

All experimental procedures were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. The experimental period occurred from October, 2014 to January, 2015. All cows were housed in tie stalls with sand bedding and fed ad libitum feed and water. The diet was formulated to meet cow’s requirements according to NRC (2001) recommendations.

Experimental design and aflatoxin challenge procedure

Detailed procedure is described elsewhere (Sulzberger et al., JDS, in press). Briefly, the research design was a replicated 5 × 5 Latin square using a total of 10 rumen-cannulated multiparous Holstein cows (BW = 1450 ± 134 kg) with 233± 56 DIM. Each cow was assigned to 1 of 5 treatments that were arranged so the carryover effect could be evaluated. Treatments were: POS, no clay plus an AF challenge; three different concentrations of clay, 0.5%, 1%, and 2% of the dietary DMI; and C, no clay and no AF challenge. The clay used in this experiment was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) and had the respective minerals content (% DM): magnesium = 7.2%, silicon = 6.3%, aluminum > 5%, iron = 6.9%, potassium = 0.5%, and manganese < 0.1%. Ion chromatography (IC) was used to report the presence of other chemical functional groups. The clay’s ion composition (PPM) was: sulfate = 124, chloride = 113, carbonate = 641, nitrate = 97, and phosphate = 2 (Avomeen Analytical Services, Ann Arbor, MI).

Periods (21 d) were divided into two phases, an adaptation phase (d 1 to 14) with regular TMR, and a challenge phase (d 15 to 17). The average DMI from d 12 to 14 was used to
calculate the amount of aflatoxin needed for dosing. Days 15 to 17 served as AF dosing days in
which a total of 100 µg / kg AFB$_1$ of DMI was fitted into gelatin capsules delivered intra-
ruminally directly after feeding for three consecutive days. All cows were fed the same TMR
throughout the trial and were fed once daily at 1400h. The clay allocation was weighed daily to
correlate to kg of TMR. Each equal allocation was mixed with 0.5 kg ground corn and top-
dressed onto the TMR at 0600 and 1400.

Liver sampling and triglycerides analysis

Liver tissue was sampled via puncture biopsy (Dann et al., 2006) under local anesthesia
(1 sample per cow per period) on d 18 during the last week of each period. All samples were
collected and stored in liquid nitrogen. For liver tissue triacylglycerol (TG) content analysis, a
total of 50 mg of tissue was first homogenized in 1.5 mL of PBS/10 mM EDTA using a handheld
homogenizer (Tissue-Tearor, Biospec Products). Subsequently, 200 µL of GPBS-142 EDTA
along with 3 mL of isopropanol-hexane-water (80:20:2 vol/vol) were added to each sample, the
tube was covered with aluminum foil, and the mixture was incubated for 30 min at room
temperature. One milliliter of hexane-diethyl ether (1:1) was then added to each sample followed
by vortexing and incubating for 10 min at room temperature (protected from light). One milliliter
of water was added to each sample to separate the lipid phase and the mixture was vortexed.
Samples were incubated covered with aluminum foil for ~20 min at room temperature. The
organic phase was then aspirated and placed into glass vials, prior to evaporation under a stream
of N gas. An 8-point TAG standard was prepared with Infinity TG reagent 205 (Cayman
Chemicals). Each 150-µL sample was mixed with 540 µL of Infinity TG reagent prior to
vortexing. A total of 160 µL of this sample mixture was pipetted into a flat-bottom 96-well
plastic microplate. The plate was incubated for 15 min at 37 °C prior to determining absorbance
at 540 nm using a microplate reader. Concentration of TAG was calculated from the standard curve.

**Hepatic Gene expression**

**RNA extraction.** Total RNA was extracted using Qiazol® reagent (Qiagen, Germany). A total of 50 mg of tissue was placed in 1 mL of Qiazol® homogenized with a Bead Beater 16 (Biospec, OK, USA) using two 30-sec cycle of the homogenizer at full speed and placed on ice after any homogenization for one minute. Homogenized sample were centrifuged to remove any remaining cell debrides. Chloroform was then added to homogenized sample, centrifuged, and aqueous phase carefully removed. Precipitation of RNA was achieved with the addition of ethanol (Deacon Lab Inc., PA, USA), and the subsequent RNA pellet was washed and cleaned using miRNeasy mini spin columns (Qiagen, Germany). Genomic DNA was removed from RNA during purification with DNase (Qiagen, Germany). The RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), while RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies). All samples had a RNA integrity value greater than 8.0.

**Primer Design and Evaluation.** Primers were designed using Primer Express 3.0 with minimum amplicon size of 80 bp (amplicons of 100–120 bp were of superiority, if possible) and limited 3’ G + C percentage (Applied Biosystems) (Table 4.1). Primer sets were intentionally designed to fall across exon-exon junctions. Then, primers were aligned against NCBI database through BLASTN and UCSC’s COW (*Bos taurus*) Genome Browser Gateway to determine the compatibility of primers with already annotated sequence of the corresponding gene in both databases. Prior to quantitative real time PCR (qPCR), primers were verified through a 20-μL PCR reaction, which followed the same procedures of qPCR described below except the
dissociation step. A universal reference cDNA amplified from all samples was utilized to ensure the identification of genes. Five microliters of PCR product was run in a 2% agarose gel stained with ethidium bromide, and the remaining 15 μL were cleaned with a QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. The sequencing product was confirmed through BLASTN at the National Center for Biotechnology Information (NCBI) database. Only primers that presented a single band of the expected size and the right amplification product were used for qPCR.

**cDNA synthesis and qPCR.** Seven genes related to the inflammatory response were selected for transcript profiling in liver tissue; albumin (ALB), haptoglobin (HP), interleukin 1 β (IL1B), interleukin 6 (IL6), nuclear factor kappa B (NFKB1), signal transducer and activator of transcription (STAT3), and tumor necrosis factor α (TNFA). A portion of the RNA was diluted to 100 mg/L using DNase/RNase free water prior to reverse transcriptase. cDNA was synthesized using 100 ng RNA, 1 μg dT18 (Operon Biotechnologies, AL), 1 μL 10 mmol/L dNTP mix (Invitrogen Corp., CA), 1 μL random primers (Invitrogen Corp., CA), and 10 μL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 6 μL of master mix composed of 4.5 μL 5X First-Strand Buffer, 1 μL 0.1 M DTT, 0.25 μL (50 U) of SuperScriptTM III RT (Invitrogen Corp., CA), and 0.25 μL of RNase Inhibitor (10 U, Promega, WI) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:4 (v:v) with DNase/RNase free water.

Quantitative PCR (qPCR) was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL SYBR Green master mix (Applied Biosystems, CA), 0.4 μL
each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA). Each sample was run in triplicate and a 6 point relative standard curve plus the non-template control (NTC) were used (User Bulletin #2, Applied Biosystems, CA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA). The final data were normalized using the geometric mean of 3 internal control genes: GAPDH, RPS9, and UXT (Khan et al., 2015).

**Statistical Analyses**

Data were log₂ normalized and then analyzed using the mixed model procedure of SAS (v 9.3; SAS Institute Inc., Cary, NC) to account for carryover effect by the following model:

\[ y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + C_m + e_{ijklm}, \]

where \( y_{ijklm} \) is the observations for dependent variables; \( \mu \) is the general mean; \( S_i \) is the fixed effect of the \( i^{th} \) treatment sequence; \( A_{(i)j} \) is the random effect of the \( j^{th} \) cow in the \( i^{th} \) sequence; \( P_{(i)k} \) is the fixed effect of the \( k^{th} \) period; \( T_l \) is the fixed effect of the \( l^{th} \) treatment; \( C_m \) is the fixed carryover effect from the previous period (\( C = 0, \) if period = 1); \( e_{ijklm} \) is the random error. Carryover effects were not detected (\( P > 0.40 \)), therefore, data were analyzed as a replicated Latin square by the following model:

\[ y_{ijklm} = \mu + S_i + A_{(i)j} + P_{(i)k} + T_l + e_{ijklm}. \]
where $y_{ijklm}$ is the observation for dependent variables; $\mu$ is the general mean. $S_i$ is the random effect of the $i^{th}$ square; $A_{ij}$ is the random effect of the $j$th cow in the $i^{th}$ square; $P_{ik}$ is the fixed effect of the $k^{th}$ period; $T_l$ is the fixed effect of the $l^{th}$ treatment; and $e_{ijkl}$ is the random error. The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers (Littell et al., 2002).

Contrasts were made using the CONTRAST statement of SAS: 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%. Back-transformed least squares means are reported with associated standard errors. Residual distribution was evaluated for normality and homoscedasticity. Statistical significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

There are known methods to detoxify aflatoxin-containing feedstuffs, however they are expensive and difficult for the typical farmer to accomplish (Kubena et al., 1998; Guo et al, 2005; Kabet and Var 2006). Thus, AF continue to be resilient and make its way to ruminant consumption. Not only do AF increase the risk of liver cancer, they also cause other adverse effects when ingested: decreased growth and performance in chickens (Fowler et al., 2015), decreased liver functionality and increased haptoglobin in bovine (Kubena et al., 1998; Queiroz et al., 2012; Xiong el al., 2015), and lowered concentrations of vitamins A and E in humans (Tang et al., 2009). Fowler et al. (2015) reported that calcium bentonite resulted in a 43.5% reduction in AFB$_1$ residue in liver tissue of broiler chickens. Various studies fed assorted clay feed additives, such as montmorillonites and sodium bentonites at different percentages, then
orally introduced AF in order to measure transfer rates of AFB\textsubscript{1} to AFM\textsubscript{1} (Kutz et al., 2009; Kissell et al., 2013; Maki et al., 2015; Xiong et al., 2015). Overall, clay supplementation ultimately reduced transfer rates of AF to milk.

Immune systems work similarly against different types of pathogens. Innate and adaptive immunity have the same structure in how to protect the body from an invader. The innate immune system works to deal with the problem at hand, and sends signals (cytokines) to relay messages to other parts of the body. Cytokines trigger releases of acute phase proteins and their signal cascades down to the genetic expression (Corrier, 1991; Bertoni et al., 2008). The objective of this study was to determine the effects of AF on hepatic gene expression of known inflammation markers in dairy cows and to determine if there was a benefit of feeding clay in the diet.

First, correlations have been made between inflammation and fatty liver for cows in the transition period (Bertoni et al, 2003; Bertoni and Trevisi, 2013). In the presence of endotoxins, Bradford et al. (2009) demonstrated that the TG storage in the liver had doubled after exposure. Moreover, late-lactation cows had an injection of the cytokine TNF-\(\alpha\) to mimic that immune response to endotoxins and confirmed the doubled storage capacity of TG in comparison to the controls. In the present study, mid-lactation cows were used, thus, fatty liver would not have accounted for any effect; furthermore, it appears that aflatoxin and clay supplementation had no effect on the TG accumulation.

Similar to endotoxins, AF are assimilated in the body through the gastrointestinal tract from which they can enter lymphatic circulation causing a proinflammatory cytokine release such as TNF-\(\alpha\), IL-1\(\beta\), and IL-6 (Bertoni et al., 2008). There were no differences for \textit{IL1B} expression, and \textit{IL6} mRNA was not detectable; however, a significant trend was found for \textit{TNFA}
(Table 4.2). Tumor necrosis factor α sends signals to activate a complex known as IKK that permits NF-κB to translocate in the nucleus through a negative feedback system (Blander, 2009; Mauro et al., 2009; Yu et al., 2009; Graugnard, et al., 2013; Minuti, et al., 2015). As tumor necrosis factor α begins the translocation process of NF-κB into the nucleus, it is logical to have both genes follow a similar linear pattern. In fact, as clay becomes increasingly introduced into the diet, $TNFA\ (P = 0.10)$ and $NFKB1\ (P = 0.02)$ were upregulated. With bacterial endotoxins, TNF-α is released as local macrophages remove the toxin (Eckel and Ametaj, 2016). For AF, as AFB1 becomes increasingly removed from the system; i.e., with added clay in the diet, it appears that the inflammatory response mechanisms are restoring its function when comparing it to the control group.

As previously mentioned, $NFKB1$ can be upregulated by a series of events, but, on the other hand, NF-κB can be affected by other indicators. Failure to enter the nucleus can happen when STAT3 is translocated into the nucleus, preventing NF-κB from being released from its complex (Blander, 2009; Mauro et al., 2009; Yu et al., 2009). To be expressed, $STAT3$ needs to interact with an intrinsic or extrinsic factor, NF-κB, and a complex known as p-300 inside the nucleus. Figure 4.1 shows the multiple interactions that occur among immune system markers in a hepatocyte (Yu et al., 2009; Blander, 2009; Eckel and Ametaj, 2016). In the present study, cows that were exposed to AF tended to have a downregulation in $STAT3$ expression. For AF to exert toxic effects it needs to bind to either DNA, RNA, or proteins that undergo a multitude of reactions to form a mutagenic species (Hsieh and Atkinson, 1990). We hypothesize that the mutagenic species formed from AF are likely interacting with STAT3, preventing translocation from happening, thus downregulating $STAT3$ gene expression.
To further understand the inflammatory status, acute phase proteins such as HP are often used in ruminant species to identify immune-challenged animals (Bertoni and Trevisi, 2013; Ogunade et al., 2016). In the present study, the expression of HP was significantly downregulated when cows were challenged with aflatoxin. During an inflammatory response that arise from pathogen(s) (e.g., *M. haemolytica*) serum HP concentrations are normally elevated (Hanthon et al., 2014). Moreover, Murray et al. (2014) associated calves higher HP with higher rectal temperatures, lesser attitudes, and higher mortality in the first 4 mo of life. However, HP is not only associated with inflammation during major stressors and diseases, but it also circulates at certain concentrations under ‘normal’ physiological states (Bertoni and Trevisi, 2013). The aforementioned studies were not involving aflatoxin. Queiroz et al. (2012) challenged cows with AF, they reported elevated serum HP when cows were fed a TMR contaminated with 75 µg/kg AFB₁. Ogunade et al. (2016) reported an increase in acid-soluble protein concentrations when cows were exposed to aflatoxins. Aflatoxin mediated immune responses are unknown when comparing bacterial toxins and viral pathogens; therefore, there is no clear definition of what is supposed to be happening. Various studies have measured aflatoxins effect on immune suppression and modulation. For example, Bruneau et al. (2012) measured macrophage functionality when exposed to low prolonged doses of aflatoxin. This resulted in a negative impact on the macrophages ability to properly stimulate cytokine release. Bakheet et al. (2016) observed decreases in IL-2, TNFα, IL-17, and IFN-γ in mice in the spleen and serum. Furthermore, Meissonnier et al. (2008) demonstrated a decrease in lymphocyte proliferation after exposure to AFB₁ in pigs. In the present study, a decrease in HP gene expression would agree with the suggestion that aflatoxin suppresses inflammatory response.
The inflammation markers examined through hepatic expression are not the only way to detect changes in inflammatory status. Ogunade et al. (2016) looked at various blood parameters such as blood cell counts, cytokines, and acute phase proteins. This study found a reduction in toxin-induced inflammatory stress when cows were fed diets containing sequestering agents including sodium bentonite. More pertinently, Mehrzad et al. (2011) studied the in vitro activity of neutrophils exposed to low doses of AFB₁. Just by focusing on neutrophil activity, they were able to determine that neutrophils exposed to AFB₁ decreased effectiveness and the production of free radicals increased. Free radicals are used by the antioxidant superoxidase dismutase (SOD) that converts superoxide to hydrogen peroxide and is the enzyme commonly involved in oxidative stress (Yuan et al. 2012). Since the liver is a major detoxification organ, the rise in serum SOD in the presence of AF is meant to better protect itself from damage (Rodriguez et al., 2004). Our group previously reported an increase in serum SOD when cows were challenged with AF that would support this finding (Sulzberger et al., JDS, in press).

**CONCLUSIONS**

In conclusion, AF has been shown to act as an immunosuppressant in many species including dairy cattle. When clay was added to the diet, the suppressive effects of AF were shown through HP and STAT3. The suggested immunosuppressive effects were alleviated when cows were fed clay supplementation. The two directly linked together, TNFα and NF-κB had increasing linear trends that coincide with the normal ranges of the control group. This finding is relevant to the dairy industry due to the high prevalence of AF worldwide and through the strict FDA regulations. It also is critical for the well-being of animals takes precedence through the transition period where cows are known to be at the most immunocompromised state.
<table>
<thead>
<tr>
<th>Accession #</th>
<th>Symbol</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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</thead>
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<td>BC151546.1</td>
<td>ALB</td>
<td>AGTGCTGCACAGAGTCATTGGT</td>
<td>GGCTTTGGGTACATATGTTTCATCA</td>
</tr>
<tr>
<td>NM_001034034.2</td>
<td>GAPDH</td>
<td>TGGAAAGGCCATCACCATCT</td>
<td>CCCACTTGATGTTGGCAG</td>
</tr>
<tr>
<td>NM_001040470.2</td>
<td>HP</td>
<td>GGTTCGGAAAAACCATCGCTA</td>
<td>CACTCGTGTCCTCCTCCCTC</td>
</tr>
<tr>
<td>EU276067.1</td>
<td>IL1B</td>
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<td>TACCCAAGGCCACAGGAATCT</td>
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<tr>
<td>BC153232.1</td>
<td>NFKB1</td>
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<td>NM_001101152.2</td>
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<td>UXT</td>
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Table 4.2 Least squares means and associated SEM for gene expression 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 (C) treatments.

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>POS</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>C</th>
<th>SEM</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Genes</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>ALB</td>
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<td>1.97</td>
<td>2.23</td>
<td>2.27</td>
<td>2.06</td>
<td>0.15</td>
<td>0.83</td>
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<td>HP</td>
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<td>1.97</td>
<td>1.44</td>
<td>1.64</td>
<td>2.52</td>
<td>0.70</td>
<td>0.04</td>
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<td>IL1B</td>
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<td>1.77</td>
<td>2.21</td>
<td>1.84</td>
<td>2.06</td>
<td>0.25</td>
<td>0.45</td>
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<td>NFKB1</td>
<td>1.78</td>
<td>1.71</td>
<td>1.86</td>
<td>2.00</td>
<td>1.87</td>
<td>0.08</td>
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<tr>
<td>STAT3</td>
<td>1.75</td>
<td>2.11</td>
<td>2.00</td>
<td>1.91</td>
<td>2.25</td>
<td>0.26</td>
<td>0.10</td>
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<td>TNFA</td>
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<td>1.76</td>
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<td>1.99</td>
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<td>TG, mg/g wet</td>
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<td>0.60</td>
<td>0.58</td>
<td>0.56</td>
<td>0.67</td>
<td>0.09</td>
<td>0.98</td>
</tr>
</tbody>
</table>

1 Dietary treatments were positive control diet [POS, without clay (0%) and with 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. AF challenge: 100 µg aflatoxin/ 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%.

ALB = albumin; HP = haptoglobin; IL1B = interleukin 1 beta; NFKB1 = nuclear factor kappa B; STAT3 = signal transducer and activator of transcription; TNFα = and tumor necrosis factor α; TG = triglycerides.
Figure 4.1 Diagram of interactions between inflammation markers in a hepatocyte cell. STAT3 activation needs an extrinsic or intrinsic factor to activate, however it has the potential to inhibit IKK release of NFKB. Thus, once that happens, STAT3 transcription would be downregulated. However, IKK can also be activated by proinflammatory signals such as TNF-α which stimulates release of NFKB and translocation happens for transcription. STAT3 transcription needs a whole complex of NFKB, AF, and p300 inside the nucleus. Photo adapted from Yu et al., 2009; Blander, 2009. AF = Aflatoxin; NFKB = nuclear factor kappa B; STAT3 = signal transducer and activator of transcription; TNF-α = tumor necrosis factor alpha; IKK = IKK kinase protein; p300 = complex.
REFERENCES


Fowler, J., W. Li, and C. Bailey. 2015. Effects of a calcium bentonite clay in diets containing aflatoxin when measuring liver residues of aflatoxin B\textsubscript{1} in starter broiler chicks. Toxins 7:3455-3464.


CHAPTER 5  
Overall Summary, Conclusions, and Perspectives

The clay product used in these studies closely resembling the structure of the smectite clays and aforementioned data and research have proven that smectite clays provide beneficial effects in rumen environment, metabolism, and immunity. When cows are challenged with a high load of grain, causing SARA, clay supplementation alleviates the impact it has on rumen environment and metabolism. Clay increased rumen and fecal pH, decreased the amount of time that the rumen pH spent below 5.6, and ultimately increased milk fat yields that have shown to decrease in SARA cases. Increasing milk fat during a SARA challenge would indicate that the rumen microbiome is balanced, however, further testing would need to be done to prove this to be true. Cows that received any combination of clay tended to yield more milk ultimately higher 3.5% FCM and ECM than cows without clay supplementation.

Our results during the aflatoxin challenge seem to indicate that this clay product does not improve or aid the cow’s efficiency or production, but does provide the body and metabolism with a more effective way of eliminating aflatoxin from the system. This was demonstrated in the lower AFM$_1$ excretion and transfer percentages found when cows were fed clay in the diet. Fecal concentrations showed a decrease in concentration indicating that the aflatoxins may be exchanging ions with the clay structure not allowing it to be identified with lab analysis. Aflatoxin did not alter concentrations of vitamins as seen in previous human studies, but it did have an effect on the serum blood chemistry tested.

Aflatoxin also showed its suppressive effects when evaluating hepatic gene expression. Other studies have shown suppressed innate immune system, cytokines and acute phase proteins, however, when looking deeper at the hepatic gene expression, there were effects from aflatoxin.
intake and clay supplementation. The altered expression in immune system markers indicate that the aflatoxin has a suppressive effect, but when clay is supplemented in the diet, it appears to have a different alleviating effect on these markers. In conclusion, when a cow is challenged or immunocompromised, clay supplementation shows promising results to provide benefits for rumen environment, production, and metabolism.