1-1-2017

Efficacy of Bentonite and Calcium Montmorillonite Clays at Reducing Aflatoxin M1 Transfer in Lactating Holsteins

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Efficacy of bentonite and calcium montmorillonite clays at reducing aflatoxin M_1 transfer in lactating Holsteins

By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agriculture
in the Department of Animal and Dairy Sciences

Mississippi State, Mississippi

August 2017

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Aflatoxins are naturally occurring carcinogens found on grains, particularly in warmer climates. Because of their carcinogenic properties, they are strictly regulated and are only allowed in minimal amounts. Aflatoxin B_1, the most potent naturally occurring carcinogen known, is metabolized in the liver to form aflatoxin M_1, which is present in the milk of lactating animals. If aflatoxin concentrations are elevated above legal limits, the milk cannot be used for human consumption. Because of this, research has been conducted to evaluate ways to mitigate its absorption in the animal and prevent transfer to the milk. One such way is through the use of clay adsorbents. The current studies aimed to evaluate the efficacy of two different clay adsorbents at preventing aflatoxin transfer to the milk of Holsteins fed a known concentration of aflatoxin.

DEDICATION

This thesis is dedicated to my parents and loving fiancé. Their constant encouragement and support has driven me to pursue my dreams.

ACKNOWLEDGEMENTS

I would like to thank Dr. Stephanie Ward and Dr. Brian Rude, who have both served as my major professor. Both Dr. Rude and Dr. Ward have challenged and guided me throughout the completion of this degree. Their guidance has given me the skills necessary to continue to push forward in my career. I would also like to thank Dr. Hart Bailey for his time and wisdom given to improve my writing and knowledge.

I would like to thank Cathy Autman for her help in analyzing my feed samples. I would also like to Dr. Sparks for analyzing my milk samples. I am also thankful for the collaboration with Dr. Phillips and Cody Maki at Texas A&M University and their help and work on my projects. I would also like to acknowledge all of the graduate students and undergraduate students that helped me throughout graduate school. I could not have completed my projects without the dedication of undergraduate students that worked with me or without the advice of my fellow graduate students.

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CHAPTER I

LITERATURE REVIEW

Mycotoxins

Advancements in animal agriculture have led to increased grain production and the use of grain to fulfill dietary requirements. Mold growth on grain is a natural occurrence, however it can have detrimental effects. Molds are fungi that that grow by producing long filaments known as hyphae, which are important for survival and dispersal of fungi, and can grow into a network known as mycelium. Grain molds may also produce spores capable of aerial dispersion (Santin, 2005). The presence of these molds may not only potentially destroy nutrient components of grain, but may also produce toxic secondary metabolites known as mycotoxins.

History of Mycotoxins

Mycotoxins have potentially plagued crops since BCE times. They were written about in the Dead Sea Scrolls, describing the destruction of "houses of mildew" (Richard, 2007). Ergot alkaloids, known as "St. Anthony's Fire" during the Middle Ages, were used as Chinese medicine over 500 years ago, but are also speculated to have contributed to the Salem witch trials in Salem, Massachusetts, due to their ability to cause gangrene and convulsions (Richard, 2007).

The fermentation ability of fungi was discovered in the late 1800s and early 1900s, leading to research about the "secondary metabolites" and the growing antibiotic

industry (Richard, 2007). This led to the discovery that some metabolites were toxic to animals, revealing that fungi were able to produce toxins dangerous to humans and other animals (Richard, 2007). This knowledge was combined with research showing grain deterioration due to fungi infestation leading to the discovery that fungi could both deteriorate grains and cause toxicities when consumed (Krska et al., 2012).

The Discovery of Aflatoxins

In 1961, previously unknown found in peanut meal were discovered to be the cause "turkey X" disease, which led to the death of numerous animals consuming diets containing certain lots of peanut meal that originated in South America (Blount, 1961). The toxic effects of the disease were discovered to be caused by the presence of *Aspergillus flavus*, and the toxins discovered from extracts of isolated fungal cultures were named "aflatoxin". The disease outbreaks in turkeys and reports of cancer found in rainbow trout fish fed peanut and cottonseed meal led to the discovery of secondary metabolites (Madrigal-Santillán et al., 2010). New knowledge of the nature of aflatoxin (AF) began a combined effort from multiple areas of science leading to the discovery of new mycotoxins, their role diseases among animals, and the beginning of modern mycotoxicology (Richard, 2007).

Although the effects of AF have potentially been observed throughout history, the minute amounts they are present in impeded their discovery until technological advances in the mid 1900s. Development of assays led to the evaluation of associating AF ingestion with incidence of hepatocellular carcinoma (Kensler et al., 2011). In 1963, aflatoxin B_1 was structurally characterized (Asao et al., 1963), leading to the studies of the mechanisms of their toxicological effects (Kensler, 2011). The output of research

resulted in the classification of AF as a human carcinogen by the International Agency for Research on Cancer in 1994 (Kensler et al., 2011).

Mycotoxigenic fungi

Fungi can commonly be divided into two groups: field (plant pathogenic) and storage (saprophytic) fungi (Placinta et al., 1999). Field fungi invade seeds before harvesting and include species of *Fusarium, Alternaria, Clodosporium, Dilodia, Gibberella,* and *Helminthosporium* (Santin, 2005). Storage fungi require less moisture than field fungi and tend to invade grains and seeds during storage. These include species of *Aspergillus* and *Penicillum* (Santin, 2005). Mycotoxigenic fungi associated with the human food chain mainly belong to three genera: *Aspergillus, Fusarium,* and *Penicillum* (Sweeney and Dobson, 1998).

Aflatoxins are considered to be the mycotoxin of greatest significance in foods and feeds, and are most commonly produced by species of *Aspergillus* (Sweeney and Dobson, 1998). *Aspergillus* species are also known to produce other mycotoxins, such as sterigmatocystin, ochratoxin A (OTA), and cyclopiazonic acid (CPA; Sweeney and Dobson, 1998; Stantin, 2005). Ochratoxin A and CPA can also be produced by some *Penicillium* species (Sweeney and Dobson, 1998). *Penicillium* species are also known to produce approximately 100 toxigenic species, including numerous mycotoxins (Pitt and Leistner, 1991), such as OTA, patulin, and citrin (Sweeney and Dobson, 1998). Numerous *Fusarium* species, as well as other fungal genera, produce trichothecenes, the most chemically diverse group of mycotoxins (Sweeney and Dobson, 1998). *Fusarium* species are also known to produce fumonisins (Sweeney and Dodson, 1998). *Fusarium*

species are most associated with cereal grains, due to decreased temperature requirement for fungal growth compared to *Aspergillus* species (Placinta et al., 1999).

Aflatoxins

Aflatoxins are secondary metabolites produced by different species of *Aspergillus, Fusarium, Penicillum, Claviceps,* and *Alternaria* (Huwig et al., 2001). Species of *Aspergillus* are most common and include *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nominus* (Sweeney and Dobson, 1998). Because *Aspergillus flavus* is considered one of the most common storage molds, AF can be found around the world (Ramos and Hernández, 1997) and in numerous plant crops (Applebaum et al., 1981). In feeds, AF mainly occur in the forms aflatoxin B_1 (AFB1), B_2 (AFB2), G_1 (AFG1), or G_2 (AFG2; Figure 1.1), although many less common forms of AF exist (Brown et al., 1998; Kilch, 2007). All forms of AF differ in both chemical structure and toxicity (Christensen and Meronuck, 1986). The letters refer to their respective fluorescence under ultraviolet light, and numbers represent their relative migration distance on a thin-layer chromatographic plate (Kilch, 2007). Aflatoxins B_1 and B_2 are characterized by the fusion of a cyclopentone ring to the lactone ring of the coumarin moiety and exhibit blue fluorescence under ultraviolet light. Aflatoxins G_1 and G_2 contain a fused lactone ring and exhibit green fluorescence under ultraviolet light (Kensler et al., 2011). However, AF contamination may not always fluoresce under ultraviolet light (Kilch, 2007).

Aflatoxin B_1 is the most toxic and abundant in naturally contaminated feeds and is also a carcinogenic compound to both humans and animals (Firmin et al., 2011). While most view exposure to contamination as the consumption of AF, exposure can also result

from skin contact (Rastogi et al., 2006), absorption through vaginal mucosa (Gallo et al., 2008), or inhalation (Jakab et al., 1994; Rastogi et al., 2006).

Due to the C_1–C_2 double bond that exists in both AFB1 and AFG1, they are considered both carcinogenic and mutagenic (Koser et al., 1988). Isolates studied by Kilch and Pitt (1988) showed an increased abundance of aflatoxins B compared to aflatoxins G. Although aflatoxins B and G were both produced in large amounts in nearly all isolates of *Aspergillus parasiticus*, aflatoxins B were much more dominantly produced by *Aspergillus flavus* compared to aflatoxins G. Aflatoxin B_1 and usually AFB2 were produced in 39 of 95 (41%) of isolates of *Aspergillus flavus*, while only 6 of 63 (9.5%) produced aflatoxins G (Kilch and Pitt, 1988).

Contamination in Feeds

Toxic concentrations of AF are commonly produced in corn, cottonseed, and peanuts, and lesser concentrations of AF can be found in small cereal grains such as oats, barley, and wheat (Pier, 1992). Ensilage may be contaminated with AF if crops used for ensilage were also contaminated, and high moisture corn is of increased risk for AF contamination because the moisture content promotes growth of AF producing fungi (Pier, 1992). Hay is rarely a source of AF contamination, however it may be a source of other mycotoxins (Pier, 1992). Aflatoxin has also been found in sausages and other meat products in Germany, rice and fish sauces in Thailand, and peanut butter in the Philippines (Christensen and Meronuck, 1986).

Aflatoxin is produced by molds invading plant tissue (Queiroz et al., 2012). The production of toxigenic strains of *Aspergillus* is dependent on the availability of moisture

and feed present on crops. Roughly 50% of *Aspergillus flavus* and *Aspergillus parasiticus* strains are toxigenic (Pier, 1992).

Fungal growth and potential subsequent AF contamination can occur in two different phases. The first phase includes the infection of the growing crop and the second includes increases in contamination after maturation (Cotty and Jamie-Garcia, 2007). Although contamination is usually attributed to one or the other, both phases may contribute (Cotty and Jamie-Garcia, 2007). Most commonly, AF contamination is a field issue rather than a storage issue, as storage conditions can be managed but field conditions are subjected to uncontrollable natural events (Christensen and Meronuck, 1986; Pier, 1992). Fungi will produce mycotoxins in the presence of stress, such as changes in temperature, moisture, aeration, or the presence of aggressive agents (Santin, 2005). Spore production in crops is dependent on elevated moisture and relative humidity in the environment (Guo et al., 1995; Brown et al., 1998). Feeds must have at least 15 % moisture content to support mold growth (Pier, 1992), and a relative humidity of 85 % is required for *Aspergillus flavus* to grow and produce AF (Christensen and Meronuck, 1986). Guo et al. (1995) reported that elevated concentrations of AF did not occur until relative humidity reached 91 % for both susceptible and resistant species of corn. For this reason, shelled corn is not often associated with an abundance of AF contamination because it is rarely stored at moisture contents above 17 to 18 % and at temperatures optimal for fungal growth (Christensen and Meronuck, 1986). However, AF producing fungi may proliferate if improperly stored. Aflatoxin contamination is more often associated with crop production in warmer climates (Cotty and Jaime-Garcia, 2007; Krska et al., 2012) and in feeds that are stored on the farm (Battacone et al., 2012). While

rainy conditions close to harvest could increase AF contamination, drought conditions may also increase AF contamination (Pier, 1992; Cotty et al., 1994; Cotty and Jamie-Garcia, 2008). Stress related to AF contamination is most commonly seen in southern states, however has been seen in the Midwest as well (Richard, 2007). When evaluating feed for contamination, it is important to note that contamination may not be evenly distributed within the feed source (Battacone et al., 2012).

Aflatoxin B_1

Only a few AF are naturally occurring, and of those AFB1 is most important (Brown et al., 1998). Aflatoxin B_1 is the most potent naturally occurring carcinogen known (FDA, 2012). Because of this, AFB1 is classified as a group 1 carcinogen, meaning it is carcinogenic to humans (IARC, 1993).

Microbes in the rumen are often a first line of defense against ingested mycotoxins. However, when the degradation of AFB1 was studied in vitro using rumen fluid collected from fistulated cattle and sheep, there was only a slight decrease in AFB1 within 30 minutes and no further decrease after (Kiessling et al., 1984).

Askit et al. (1997) evaluated the exposure to AF of infants fed breast milk and formula. The frequency of serum AFB1 was greater in formula fed infants compared to infants fed breast milk, although serum AFB1 concentrations in mothers of both infant groups were not different. This prompted an investigation of AFB1 concentrations of commercial infant formulas. The 8 most commonly used commercial infant formulas were evaluated and AFB1 concentrations were determined on the first day of opening, as well as on d 15 (maximum use date recommended by manufacturers) and d 30 after opening. Packages were stored at room temperature and were opened 5 times daily and

stirred with spoons to allow the formula to contact room air. Although they were all within legal concentrations, 7 of the 8 infant formulas were positive for AFB1 when first opened (Table 1.2). In addition, AFB1 concentration was greater on d 30 than on d 15 and d 0. There was no difference between d 15 and d 0, however AFB1 concentrations were above legal limits by d 15 in some formulas. Askit et al. (1997) recommended that infant formula be stored in a refrigerator at 4°C, rather than room temperature, to protect it from humidity, and to restrict use to 15 d after opening.

Aflatoxin M_1

Aflatoxins M_1 (AFM1) and M_2 (AFM2) are the monohydroxylated derivatives of AFB1 and AFB2. In the liver, AFB1 is metabolized through the cytochrome P450-mediated oxidation producing less toxic and more water-soluble metabolites such as AFM1 (Figure 1.2). Its letter refers to its presence in milk. Aflatoxin M_1 concentration is directly correlated with AF consumption. Aflatoxin consumption increases AFM1 secretion in milk (Battacone et al., 2003; Battacone et al., 2005; Battacone et al., 2012; Maki et al., 2016a; Maki et al., 2016b), curd (Battacone et al., 2005), and whey (Battacone et al., 2005).

When consumed by humans AF is considered potentially carcinogenic, classifying it as a group 2B carcinogen (IARC, 1993). Contamination of AFM1 has been observed in numerous food products including infant formula, dried milk, cheese, yogurt, and in milk products from various animals, including in human breast milk (Galvano et al., 1996).

Absorption and secretion of aflatoxins

Absorption and metabolism of AFB1 occurs rapidly. Gallo et al. (2008) reported that AFB1 was detectible in blood plasma of dairy cows at 5 min and peaked as soon as 20 min after consumption of an oral bolus containing 4.9 mg of AFB1. The same study showed that AFM1 was detectable in the blood at 5 min and peaked at 25 min after consumption of the bolus. A second trial from Gallo et al. (2008) reported that both AFB1 and AFM1 were detectable in blood plasma at the first collection period (15 min), and AFB1 peaked in the blood at 30 min after being given a vaginal implant containing 4.89 mg AFB1. The same trial reported the greatest concentration of AFM1 in the first milking post administration of vaginal implant. Aflatoxin M_1 was detectible in milk of dairy goats 1 h after consumption of AFB1, and peak AFM1 concentration in milk occurred 3 to 6 h after consumption of AFB1, and then rapidly declined (Battacone et al., 2012). These results were consistent with that of a previous study by Battacone et al. (2003) with ewes.

Aflatoxin M_1 is removed from the system rapidly as well. After AFM1 concentrations peaked, they rapidly decreased, and after 84 h were no longer detectable in the milk of dairy goats fed 0.8 mg of pure AFB1 (Battacone et al., 2012). Concentrations of AFM1 were no longer detectable after 72 h from the last treatment in dairy ewes administered AFB1 for 8 d (Battacone et al., 2003). In dairy cows, AFM1 concentration decreased close to initial values at 36 hours post administration of a vaginal implant containing 4.89 mg of AFB1 (Gallo et al., 2008). Moschini et al. (2008) reported the greatest concentration of AFM1 was detected in the first milking after cows were drenched with 300 mL of 0.367 µg AFB1/mL solution.

Aflatoxin M_1 Stability in Milk

Processing of milk has variable results on AFM1 concentration. Purchase et al. (1972) reported a 32% reduction of AFM1 after pasteurization at 62°C for 30 min, with a decrease in concentration as pasteurization increased in both temperature and time. In contrast, pasteurization of milk at 63°C for 30 min resulted in no change of AFM1 contamination in a study by Stoloff et al. (1975). When making cottage cheese, Stoloff et al. (1975) reported a 20% reduction of AFM1 during the process, which was much less than the near 60% reduction reported by Purchase et al. (1972). Purchase et al. (1972) reported undetectable concentrations of AFM1 in cheese, but remaining AFM1 was detected in whey. Similarly, 86% of remaining AFM1 was found in whey rather than cheese curds from the study done by Stoloff et al. (1975). López et al. (2001) artificially contaminated milk with AFM1. The contaminated milk was used to make whey and cheese, and an observed 60% of the AFM1 was detected in whey and 40% in milk. This is supported by Battacone et al. (2005), who reported AFM1 contamination in milk, whey, and cheese of11 ewes administered either 32, 64, or 128 µg AFB1/d.

Toxicity

Aflatoxicosis is the result of toxicity associated with consuming AF and may occur in acute and chronic forms (Williams et al., 2004). Acute toxicity affects numerous liver functions and may eventually result in death. Rabbits and ducks are more susceptible to acute toxicity due to their ability to rapidly metabolize AF compared to sheep and rats that metabolize AF slower. Chronic exposure to aflatoxins above legal regulations most commonly affects the liver, leading to the potential development of liver cirrhosis and cancer (FDA, 2012). Aflatoxins may also cause bleeding lesions and

cancers in organs other than the liver, such as the kidney, adrenal glands, ovary, and stomach (Khatun et al., 2012). Postmortem examination of calves administered AF showed loss of liver color, adrenal hyperplasia, accumulation of fat and loss of glycogen in the liver, and disorganized liver lobules and invasion by reticular fibers (Lynch et al., 1970). Although it is difficult to prove AF is the source of symptoms, research has supported its risk to both human and animal health unless strictly regulated (FDA, 2012). Although no outbreaks have been reported among humans in the United States, and it is a rare diagnosis in domestic animals, acute and chronic aflatoxicosis is common in both children and adults in some developing countries (FDA, 2012).

Regulations

Aflatoxin contamination is considered by the US Food and Drug Administration (FDA) to be unavoidable, so regulations have been implemented to minimize contamination (Williams et al., 2004). Due to the quantity of milk consumed and the negative effects of AF that carry over to humans consuming milk, strict regulations are in place to manage concentrations of AF allowed in animal feedstuffs as well as in milk (Table 1.2). In the United States, the FDA has established an action limit of 0.5 and 20 ppb for AF in milk and lactating cow feeds respectively (FDA, 2000). However, these regulations may be difficult to achieve because molds producing AF have the ability to infest crops both before and after harvesting (Richard et al., 2009).

Practically speaking, it is unlikely to feed contaminated feedstuffs in production, however due to the FDA regulations, a single incident of accidental feeding may force milk AFM1 concentrations above legal limits (Battacone et al., 2003). This was observed

in a study by Maki et al. (2016a) that revealed detectable AFM1 concentrations in milk of cows fed control diet, showing the presence of naturally occurring AF in the control total mixed ration (TMR).

Effects of Aflatoxin on Production

Results from research are indicative of variable effects on daily production of lactating cows from mild exposure to aflatoxins. Dry matter intake does not appear to be affected by AF consumption (Applebaum et al., 1982; Kutz et al., 2009; Queiroz et al., 2012; Maki et al., 2016a; Maki et al., 2016b), however Stroud (2006) reported a reduction of dry matter intake (DMI) from 24.0 to 22.5 kg/d by cows fed AF contaminated feeds.

Applebaum et al. (1982) reported a decrease of milk yield averaging 2.4 kg per day when cows were fed impure AFB1. In contrast, numerous investigators have reported no difference of milk yield in cows fed AF compared to those not fed AF (Stroud, 2006; Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). Battacone et al. (2005) reported no difference of milk yield by dairy ewes fed 32, 64, or 128 ≥ µg of pure AFB1 compared to control animals fed no AFB1. Battacone et al. (2003) administered a pellet to lactating ewes containing no AFB1 and were administered a pellet containing 0, 0.767, 1.597, or 3.303 µg of artificially contaminated AFB1/kg body weight (BW). The authors reported an increase in milk yield of ewes administered 1.597 and 3.308 µg of AFB1 compared to ewes administered 0 and 0.767 µg AFB1/kg BW.

Queiroz et al. (2012) reported a suppression in milk protein percent and milk fat yield by cows fed 75 ppb AFB1 compared to control animals. This study contradicts

other studies that showed no change of composition due to the consumption of AF contaminated feed (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzeberger et al., 2017). The results reported by Queiroz et al. (2012) also contradict studies done using dairy ewes which showed no change of fat and protein percent in milk (Battacone et al., 2003; Battacone et al., 2005), lactose percent in milk (Battacone et al., 2003), or fat and protein percent in curds and whey (Battacone et al., 2005).

Young pigs fed AF contaminated diets exhibited roughened hair coats, a decrease in feed intake and feed conversion ratio, and a decrease in weight gain compared to those fed control diets (Thieu et al., 2008). Broilers exhibited a decreased ADG and serum protein concentration when AFB1 is consumed (Juan-juan et al., 2010).

Economic Cost of Mycotoxins

Mycotoxin contamination is associated with increased economic costs because contaminated crops may have to be destroyed. To maintain regulations within allowable concentration of AF, an estimated $500 million to $1.5 billion is spent on crop loss, research, and monitoring activities (Robens and Cardwell, 2003). Additionally, if AF is detected in the milk above the action limit, the discard of the contaminated milk results in a loss of profit from either the dairy producer or the company supplying the contaminated feed. If a farmer chooses to include an additive in the TMR in an effort to reduce the contamination in the milk below the action limit, the feed additives represent another cost incurred by the farmer. Annual losses from mycotoxins such as aflatoxin, fumonisin, and vomitoxin are estimated to range between $0.5 million to over $1.5 billion, and of the costs associated with mycotoxins, AF is associated with the greatest management cost due to its toxicity and strict regulation. Cost of AF testing ranges between $30 and $50

million per year (Robens and Cardwell, 2003). The 2003 Council for Agricultural Science and Technology Mycotoxin Report concluded that major research and policy is needed in the "economics of mycotoxin contamination" (Wu et al., 2004).

Preventing Contamination

Genetically engineering crops for resistance to AF producing molds, such as *Aspergillus flavus*, is being evaluated for efficacy. When comparing a corn population known to be resistant to *Aspergillus flavus*, GT-MAS:gk, and a more susceptible Pioneer hybrid 3154 during preharvest conditions, increased AF concentrations were not detected until relative humidity exceeded 90% (Guo et al., 1995). When relative humidity was between 91 and 100%, AF concentrations in GT-MAS:gk averaged 98% less than concentrations in Pioneer 3154. The same study evaluated the effects of incubating kernels on contamination. While AF concentrations remained unchanged in GT-MAS:gk kernels, incubating Pioneer 3154 kernels for 3 days at 100% relative humidity resulted in a 61% decrease of AF contamination. Because of these results, eight susceptible corn species (Asgrow RX 899, Dekalb 689, Deltapine G-4666, McCurdy 7477, Oro 188, Oro 200W Pioneer hybrid 3154, and Pioneer hybrid 3165) were incubated for 3 days at 100% relative humidity. Reduction of AF contamination averaged 83%, and AF reduction within the hybrids ranged from 68 to 96%. In fact, preincubated Deltapine G-4666 kernels was similar to the resistant GT-MAS:gk kernels. Guo et al. (1995) stated that this suggests an inhibitor of aflatoxin biosynthesis can potentially be induced during the germination of kernels.

Inhibiting growth of AF producing fungi can be an effective way to decrease AF contamination in crops. Two varieties of Thyme (*Thymus eriocalyx* and *Thymus x-porlock*) resulted in decreased growth of *Aspergillus parasiticus* and may be a potential substitution to current antifungal compounds used (Rasooli and Abyaneh, 2004).

Detoxification of Crops

Postharvest treatments are administered in an attempt to keep aflatoxin concentrations within regulations. These treatments have been evaluated as a way to detoxify feeds and include thermal inactivation, mechanical and density separation, solvent extraction, biological inactivation, chemical detoxification, and sequestering agents. However, these tend to be costly and only partially effective (Kutz et al., 2009). For instance, alkaline treatments hydrolyze the lactone ring of AFB1 but reformed under acidic conditions and reformed AFB1, and the addition of sodium bisulfate is effective but reduces palatability, making it impractical (Waltman, 2008).

Mechanical Separation

Because aflatoxins are produced by mold growth, most commonly *Aspergillus flavus*, the discoloration caused by mold growth is an indicator of aflatoxin contamination (Dickens and Whitaker, 1975). For years, farmers have used mechanical separation to decrease the abundance of aflatoxin in crops. Peanut producers, for example, remove discolored kernels using electric color sorters and hand picking to decrease contamination (Dickens and Whitaker, 1975). Dickens and Whitaker (1975) evaluated the efficacy of these separation methods, and found hand picking to be more effective than electronic color sorting, removing an average of 72% of contaminated kernels, while electronic

sorting was variable. Hand picking is the simplest method of removing contamination, however it is a time-consuming process (Bata and Lásztity, 1999).

Density Separation

Aflatoxin contaminated feeds have been found to have a decreased specific gravity than those that are uncontaminated, making it possible to separate for contamination using flotation practices (Henderson et al., 1989). Using this, feeds that sink in their relative medium are generally AF-free or contain decreased concentrations of AF, and can be dried and processed, while those that float contain increased concentrations of AF (Henderson et al., 1989).

Biological Inactivation

Lactic acid bacteria have been proven to decrease toxicity of AF species. *Lactobacillus rhanosus* strains GG and LC-705 decreased AFB1 concentration by 54% and 44%, respectively, in the duodenal tissue of chickens (El-Nezami et al., 2000). The same study showed a 74%, 63%, and 37% reduction in uptake of AFB1 by intestinal tissue when in the presence of *Lactobacillus rhamnosus* strain GG, *Propionibacterium freundereichii* ssp. *Shermanii JS,* and *Lactobacillus rhamnosus* strain LC-705 respectively. Haskard et al. (2001) also found *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* strain LC-705 to be most effective at removing AFB1 from solution and more stable compared to other *Lactobacillus* strains tested. The same study also found that the binding of AFB1 to lactic acid bacterial strains was reversible through bacterial washing, and they believed this was because AFB1 is bound to bacteria by weak noncovalent interactions.

Solvent Extraction

Haskard et al. (2001) was able to recover between 87 and 96% of AF bound to different *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* strain LC-705 by extraction with chloroform. Stahr and Obioha (1982) reported a 90% reduction of AF utilizing methanolic extraction by blending corn with the solvent for 3 minutes. The authors reported that chicks fed decontaminated corn exhibited no signs of aflatoxicosis, compared to fatty degenerated livers observed in chicks fed nondetoxified corn. Additionally, methanol may be recycled for additional extractions if the AF were removed by carbon or distillation of the methanol (Stahr and Obioha, 1982).

Chemical Detoxification

Ammoniation refers to the treatment with ammonia in solution, the gaseous phase, or with substances that release ammonia to detoxify feeds (Piva et al., 1995). If adequately exposed to ammonia, AFB1 can be irreversibly altered, however, if the exposure time is not sufficient, the contaminant may revert back to its former state (Piva et al., 1995). Feeds treated with ammoniation may develop an undesirable brown color, an increase in total nitrogen and non-protein nitrogen, a decrease of nitrogen solubility, and a decrease of some amino acid content (Piva et al., 1995). Additionally, residual ammonia in the feed may be toxic and result in deterioration of animal health (Huwig et al., 2001).

Treatment with sodium bisulfate is more cost effective than ammoniation. Hagler et al. (1982) found that complete destruction of AFB1 can be achieved by soaking whole-kernel corn in 10% sodium bisulfate for 72 hours and then incubating the corn in sealed plastic bags at 50°C until day 21. However, their study also revealed that AFB2 is more

resistant (Hagler et al., 1982). Sommartya et al. (1988) reported 100 ppm of sodium bisulfate as a food preservative to be an effective method of detoxifying ground peanut kernels.

Thermal Inactivation

Although aflatoxins are heat stable mycotoxins, some success has been realized by roasting. Yazdanpanah et al. (2005) reported a reduction of AFB1 ranging between 19 and 66% and a reduction of AFB2 ranging between 17 and 63%. While the greatest reduction occurred when samples were roasted at 150°C for 120 minutes, the samples became burned and were unpalatable, therefore optimum results occurred when samples were roasted at 150°C for 30 minutes (Yazdanpanah et al., 2005). Results were similar to those reported by Conway et al. (1978) where corn contaminated with AFB1 was roasted in two commercial continuous roasters, one electrically heated and the other gas-fired. This study reported a reduction of AFB1 in contaminated corn was reduced by 40 to 81% after one passage through a continuous roaster. Greater reductions were observed when temperature was increased from 145 to 165°C. It is important to note, however, that only one sample was reduced below the legal concentration of 20 ppb. A second study was done to evaluate the effect on AFB1 reduction of treating corn at 17 to 18% moisture with 1.5% ammonia for 14 and then roasting. Reduction of AFB1 ranged from 57 to 99% (Conway et al., 1978).

Sequestering agents

A practical and effective sequestering agent must reduce bioavailability of aflatoxins without sacrificing production or nutritional content (Maki et al., 2016a). Feed

additives proven to decrease bioavailability of aflatoxins include yeast-derived products (Juan-Juan et al., 2010; Firmin et al., 2011), clays, and activated carbons and charcoals (Diaz et al., 2003). Clays may include Na and Ca bentonite (Diaz et al., 2004; Carraro et al., 2014), montmorillonite (Marroquín-Cardona et al., 2011, Querioz et al., 2012; Maki et al., 2016a; Maki et al., 2016b), and hydrated sodium-calcium-aluminosilicate (HSCAS; Kutz et al., 2009).

Activated charcoal has been used as an antidote against poisoning since the 19th century, however variable results have been reported using activated charcoals to mitigate AF, which are potentially due to its unspecific nature of binding, making it likely for essential nutrients to be adsorbed as well (Huwig et al., 2001).

Research has shown that adding adsorbent clays to diets daily is effective at minimizing aflatoxicosis in livestock. A study by Queiroz et al. (2012) evaluated the addition of two doses of commercial montmorillonite HSCAS clay-based mycotoxin adsorbent and its affect on AFM1 concentrations in dairy cows fed feed contaminated with AFB1. Results showed that adding the absorbent at 0.2% TMR dry matter (DM) prevented adverse effects of feeding AF contaminated feed, but AFM1 concentrations in milk were not decreased until the absorbent was increased to 1% DM of the TMR. Sulzberger et al. (2017) administered 100 ppb AFB1 via a 10 mL gelatin in the rumen through a rumen-cannula of Holsteins, and three doses of adsorbent clay were administered. Results showed a reduced AFM1 concentration and a decreased number of positive SNAP tests with the addition of adsorbent clay. In addition, reduced AFB1 concentrations in fecal and rumen samples were reported in cows administered clay adsorbents. Pigs fed 0.4% and 0.5% bentonite clay exhibited a performance, feed

efficiency, and blood profile similar to that of control animals fed no AF when compared to animals fed AF contaminated diets with no bentonite (Thieu et al., 2008). An in vitro study found that HSCAS bound 97.69% of AFB1 when in solution for 10 min, and binding rate remained over 96.03% for 60 min at pH 8.0 (Juan-Juan et al., 2010). The same study showed HSCAS to be more effective than yeast cell extracts and a combination of HSCAS and yeast cell extracts, however all products did exhibit binding ability (Juan-juan et al., 2010). Kutz et al. (2009) reported a reduction of transfer of AF to milk when cows were administered HSCAS adsorbents, however administration of a yeast cell culture with some HSCAS adsorbent clay did not reduce AF transfer to milk compared to cows administered no clay. Carraro et al. (2014) treated bovine milk contaminated with up to 80 ng/L (0.08 ppb) AFM1 with six different smectite rich bentonite clays, resulting with a 70 to 100% adsorption rate, while maintaining milk composition.

In a farm trial using dairy cows, NovaSil Plus (NSP), a calcium montmorillonite clay, reduced the transfer of AF from blood to milk from 1.07% to 0.52 and 0.32 ± 0.08% and total secretion was decreased from 24.38 µg/d to 11.86 and 7.38 ± 1.71 µg/d with NSP included at 0.58% and 1.17% of DMI, respectively (Maki et al., 2016a). Total secretion of AFM1 was also reduced with the inclusion of NSP, which was expected due to the decrease in transfer to milk (Maki et al., 2016a). Another farm trial resulted in a reduction of AFM1 secreted in milk with the addition of NSP (Maki et al., 2016b). Aflatoxin M_1 secreted in milk was reduced by 68% and 55% with the inclusion of 12.1 g/kg and 6.0 g/kg NSP in the diet respectively. Because the laboratory conditions (pH = 6.5) done by Marroquín-Cardona et al. (2011) was similar to ruminal pH, Maki et al.

(2016a) suspected that the adsorption of AF may take place in the rumen leaving it unavailable in the small intestine.

Binding efficacy of adsorbents is partially dependent on pH. NovaSil Plus has proven to be effective at adsorbing AFB1 at pH 6.5 in a laboratory setting (Marroquín-Cardona et al., 2011). In vitro studies by Juan-juan et al. (2010) observed increased stability of adsorbent-AFB1 complexes at pH 6.0 and 8.0 compared to pH 2.0. An in vitro study by Moschini et al. (2008) was performed to mimic how sequestering agents adsorb to AFB1 in the rumen. When the AF:Sequestering agent ratio was 1:50,000, 70 and 78% of AFB1 was adsorbed by an aluminosilicate (Novasil^{TM} Plus) in rumen fluid from lactating and dry cows respectively. At a 1:500,000 ratio of AF:Sequestering agent was used, Novasil^{TM} Plus adsorbed 98 and 99% of AFB1 in rumen fluid from lactating and dry cows respectively. An aluminosilicate (Atox) was also tested, and outperformed Novasil^{TM} Plus. When AF:Atox was 1:50,000, 86 and 90% of AFB1 was adsorbed in rumen fluid from lactating and dry cows respectively. When AF:Atox was 1:500,000, 100 and 99% of AFB1 was adsorbed in rumen fluid from lactating and dry cows respectively.

Effects of Adsorbents on Production

Clay adsorbents have been reported to decrease the transfer of AFM1 to milk without interfering with production. No change of body condition score (BCS), BW (Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017), DMI, milk yield, milk composition (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017), vitamin A and riboflavin concentration (Maki et al., 2016b) or mineral concentration (Maki et al., 2016a) in milk when clay adsorbents were supplemented to

lactating dairy cows. Queiroz et al. (2012) reported an increase of milk protein percent by ewes consuming AFB1 contaminated diets when adsorbent clay was increased from 0.2 to 1.0% DMI while control animals were intermediate.

Safety of Clay Adsorbents

A short-term study by Wang et al. (2005) reported that NovaSil clay (NS) appears to be a relatively safe when included into human diets. The study utilized 50 healthy adults administered one of two doses of NS (1.5 and 3 g/d) for a 2 wk period. Participants reported mild GI effects including: abdominal pain (6%, 3/50), bloating (4%, 2/50), constipation (2%, 1/50), diarrhea (2%, 1/50), and flatulence (8%, 4/50). No difference of reported adverse affects was observed. Additionally, authors reported no differences of hematology, liver and kidney function, electrolytes, vitamins A and E, and minerals in either group.

Detection of AFM1

AFM1 concentrations can be determined using either direct competitive enzyme-linked immunosorbent assay (ELISA) or high-performance liquid chromatography (HPLC). Recovery of AFM1 in pasteurized milk ranged from 88.0 to 106.5% and 103.0 to 120.0% for ELISA and HPLC respectively (Kim et al., 2000). Using raw cow's milk, Velasco et al. (2003) reported AFM1 recover rates of 74.6 to 109% and 80.7 to 97.9% for ELISA and HPLC respectively. In addition, results were similar when repeated with samples frozen for a maximum of 30 days (Velasco et al., 2003). Markaki et al. (1997) evaluated AFM1 recovery using ELISA followed by HPLC if the samples contained more than 5 ng/l. Using this protocol, they determined that ELISA was very reliable,

particularly for minimal contamination, but due to the small detection limit, not following with HPLC could result in reducing the detection limit by half. In addition, HPLC recovery of AFM1 was close to 100% for this study.

Summary

Aflatoxins are secondary metabolites produced predominately by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and are immunosuppressive, anti-nutritional, mutagenic, and carcinogenic. The four naturally occurring AF are AFB1, AFB2, AFG1, and AFG2, and AFB1 is considered the most potent naturally occurring carcinogen known. Exposure to AF may also occur through consumption of metabolites, such as AFM1, which is converted from AFB1 in the liver and found in milk of animals consuming contaminated feeds.

Contamination of AF is a global issue, but is strictly regulated in the United States due to its toxic and carcinogenic effects. The legal limit for AFB1 in feeds and AFM1 in milk is 20 and 0.5 ppb for livestock feeds and dairy products, respectively. To keep AF concentration below these limits, mitigation efforts have been evaluated to reduce the transfer of AF to the milk. One such method is the inclusion of adsorbent clays in contaminated diets. Previous research has reported positive results with the inclusion of clay adsorbents.

Objectives

Two studies were performed to evaluate the efficacy of clay adsorbents at preventing AFM1 transfer to milk. The objectives of the first study were to evaluate the efficacy of Mycoad, a bentonite clay with greater than 80 % smectite content (Special

Nutrients, Miami, FL), at reducing transfer of AF from the blood to the milk of lactating dairy cows and to evaluate its effects on milk production and composition. The objectives of the second study were to evaluate the efficacy of NSP at concentrations less that 0.5% DM of TMR (the smallest concentration previously tested) at preventing transfer of AF from blood to milk and its effects on milk production and composition.

Table 1.1 FDA's action levels for aflatoxins in human and animal foods[a]

Product or animal	Total aflatoxin action level (ppb)
Human food	20
Milk	0.5
Beef cattle	300
Swine over 100 lbs	200
Breeding beef cattle, swine, or mature poultry	100
Immature animals	20
Dairy animals	20

[a]From Wu et al., 2004; *Source*: FDA, 2000.

Table 1.2 Changes in aflatoxin B_1 concentrations (ppb) of 8 commercial infant formulas in relation to time after opening and storage at room temperature.

Formula	At onset	On day 15	On day 30
1	0.042	0.060	0.135
2	0.422	0.450	0.486
3	0.253	0.265	0.540
4	0.084	0.324	0.650
5	0.000	0.000	0.000
6	0.169	1.210	1.290
7	0.056	0.100	0.216
8	0.253	0.650	0.810

[a]From Askit et al., 1997

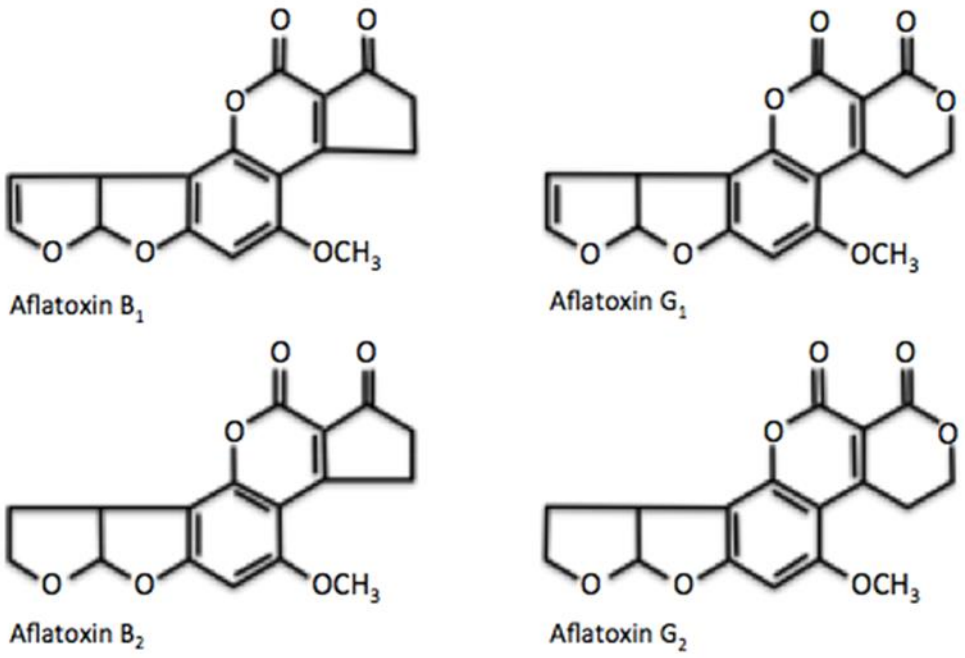

Figure 1.1 Chemical structures of AFB1, AFB2, AFG1, and AFG2 (FDA, 2012).

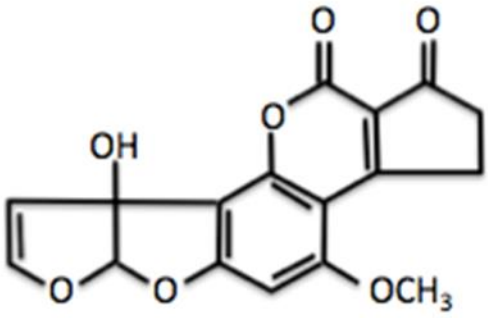

Figure 1.2 Chemical structure of AFM1 (FDA, 2012).

CHAPTER II

MATERIALS AND METHODS

Experiment One

Experimental Design and Management of Cows

This study was conducted at the Mississippi Agriculture and Forestry Experiment Station, Joe Bearden Dairy Research Center (Starkville, MS). Cows were trained to use individual feeding gates (Calan Broadbent Feeding System, American Calan, Northwood, NH) prior to treatment. Cows were housed in a free-stall pen with sand bedding. Cows were individually fed at 0530 and 1730 h, allowing for ad libitum intake, and milked at 0400 and 1600 h in a double eight parallel milking parlor.

Twenty-four mid- to late- lactation Holstein cows were used in a randomized complete block design. Cows were blocked by parity, stage in lactation, and milk production based on previous records. The experiment consisted of a 7 d treatment period. Mycoad, a bentonite clay with greater than 80 % smectite content (Special Nutrients, Miami, FL) was tested for efficacy. Intake was recorded during the training period, and was used to estimate DMI. Cows were administered 7000 µg AFB1 to target 300 ppb AFB1 in the diet, and 50 g Mycoad clay was added to respective diets, resulting in a 0.17% inclusion rate of Mycoad. Cows were randomly assigned to 1 of 4 dietary treatments (n=6): (1) control (**CON**), basal TMR with no AF or Mycoad; (2) AF (**AF**), CON plus 300 ppb AF; (3) Mycoad (**MYC**), CON plus 50 g Mycoad; (4) Mycoad clay

with AF (**MYC+AF**), CON plus 50 g Mycoad and 300 ppb AF. All additions to basal TMR were top dressed and mixed into approximately the top third of feed offered.

Feed sample and analysis

Feed and orts were sampled by treatment. Feed samples were dried at 65°C to determine air DM. Samples were then ground through a 2mm screen in a Thomas Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) and stored at room temperature. All feed samples were subjected to proximate analysis and analyzed for total DM (method 934.01; AOAC, 2009), ash (method 942.05; AOAC, 2009), crude protein (method 2001.11; AOAC, 2009), NDF (method 973.18; AOAC, 2009), and ADF (method 2002.04; AOAC, 2009; Table 2.1).

Milk sample and analysis

Milk samples were taken daily at both milkings throughout the treatment period. Samples from the 0400 h milking on d 1 and 3 of the treatment period were analyzed for fat, protein, solids, and somatic cell counts (SCC) through Mid-South DHIA Laboratories (Missouri), and results were averaged. Broad Spectrum Microtabs II™ tablets (Weber Scientific®, Hamilton, NJ) containg 8 mg Bronopol and 0.30 mg Natamycin were added to these samples immediately after collection for preservation of samples. All other samples were frozen immediately after collection. Components were analyzed using a Fourier Transform Spectrometer (Bently FTS, Bently Instruments, Chaska, MN) at Mid-South DHIA Laboratories (Missouri). Somatic cell counts were analyzed using flow cytometry (Somacount FCM, Bently Instruments, Chaska, MN). Milk component yields were calculated by multiplying the concentration of milk components by milk yield.

Samples from each milking were analyzed for AFM1 concentration according to AOAC methods. Briefly, 5 mL of milk was combined with 10 mL of acetonitrile and vortexed for 1 min. Quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction packets (Phenomenex Inc., Torrance, CA) were added, and the sample was vortexed for 1 min. Samples were then centrifuged for 5 min at 1500 x g. The supernatant was collected and analyzed using HPLC. Aflatoxin secretion and transfer were calculated. Aflatoxin secreted represents the calculated amount of AFM1 present in the milk and was determined by multiplying AFM1 concentration by milk yield. Aflatoxin transfer represents the amount of AFM1 present in the milk compared to the amount of AFB1 fed daily and was determined by dividing AFM1 secreted by AFB1 intake and multiplying by 100.

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

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

2.1

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS® (version 9.4, SAS Institute Inc., Cary, NC). Treatment, DIM, and period were considered independent variables, and milk yield, DMI, AFM1, and milk composition were dependent variables. Means were separated using Fisher's Least Significant Difference, and significance was declared when $P \leq 0.05$. Tendencies were discussed when $0.05 < P \leq 0.10$. All data were presented as mean ± the largest standard error of the mean (SEM). Data from one cow consuming the CON diet were omitted as a result of abnormal intake, and milk and milk

components yield data from another cow consuming the CON diet were omitted due to reduced milk yield and DMI. Removal of the respective cows was determined through performance of an outlier test.

Experiment Two

Experimental design and management of cows

This study was conducted at the Mississippi Agriculture and Forestry Experiment Station, Joe Bearden Dairy Research Center (Starkville, MS). Cows were trained to use individual feeding gates (Calan Broadbent Feeding System, American Calan, Northwood, NH) prior to treatment. Cows were housed in a free-stall pen with sand bedding. Cows were individually fed at 0530 and 1730 h, allowing for ad libitum intake, and were milked at 0400 and 1600 h in a double eight parallel milking parlor. Treatment was received once daily during the 0530 feeding.

Fifteen lactating Holstein cows were used in a triplicate 5 x 5 Latin square. The experiment consisted of five 10-d periods. Treatment was applied d 1 through 5 and d 6 through 10 were used as a washout period to prevent carry-over effects. NovaSil Plus was tested at different concentrations. NovaSil Plus was fed at 0.125, 0.25, and 0.5% (the smallest concentration previously tested) of the predicted DMI. Cows were fed 1652.44 µThe AF fed was produced through rice fermentation by *A.* parasiticus NRRL 2999 according to Shotwell et al. (1966) and modified by West et al. (1973). Rice powder contaminated with 758 mg AFB1/kg weight was obtained from the Food and Feed Safety Research Facility, USDA/ARS (College Station, TX), and AFB1 concentration was verified by the Office of The Texas State Chemist, Texas A&M University (College Station, TX). Dry matter intake was estimated using the DMI observed during experiment

1. Cows were randomly assigned 1 of 5 dietary treatments (n=3): (1) positive control (**CON**), basal TMR with no AF or NSP; (2) AF Control (**AFC**), CON plus 50 ppb AF; (3) NSP Control (**NSPC**), CON plus 0.5% estimated DMI NSP; (4) low-dose clay with AF (**NSP-125%**), CON plus 0.125% estimated DMI NSP and 50 ppb AF; or (5) high-dose clay with AF (**NSP-25%**), CON plus 0.25% estimated DMI NSP and 50 ppb AF. All additions to basal TMR were top dressed and mixed into approximately the top third of feed offered.

Body Condition Score

Body condition score was monitored throughout the study. One observer recorded BCS on d 4 of the treatment period, and the same observer determined BCS throughout the study. Body condition score will be measured in 0.25 unit increments on a 1 to 5 scale (Ferguson et al., 1994).

Feed sample and analysis

Basal TMR and individual orts were sampled on d 4 of each treatment period. If a sample was unable to be collected on d 4, it was collected on d 5 of the treatment period. Feed samples were dried at 65°C to determine air DM. Samples were then ground through a 2mm screen in a Thomas Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) and stored at room temperature. Subsamples of orts were taken and combined by treatment and period. All feed samples were subjected to proximate analysis and analyzed for total DM (method 934.01; AOAC, 2009), ash (method 942.05; AOAC, 2009), crude protein (method 2001.11; AOAC, 2009), NDF (method 973.18; AOAC, 2009), and ADF (method 2002.04; AOAC, 2009).

Milk sample and analysis

Milk samples were taken at both milkings on d 4 and 5 of treatment periods. Two samples were taken, and a broad Spectrum Microtabs II™ tablet (Weber Scientific®, Hamilton, NJ) containg 8 mg Bronopol and 0.30 mg Natamycin was added to one sample per cow at the 0400 h milking immediately after collection for preservation. These samples were analyzed for fat, protein, solids, and SCC through Mid-South DHIA Laboratories (Missouri), and results were averaged. Fat, protein, and solids were analyzed using a Fourier Transform Spectrometer (Bently FTS, Bently Instruments, Chaska, MN) at Mid-South DHIA Laboratories (Missouri). Somatic cell counts were analyzed using flow cytometry (Somacount FCM, Bently Instruments, Chaska, MN).

All other samples were frozen immediately after collection, and one sample per cow from each milking during the collection period were analyzed for AFM1 concentration at Texas A&M University (College Station, TX). Aflatoxin was extracted from samples (AOAC Method 2000.08; AOAC, 2000) and analyzed by LC-MS/MS (Waters H-class UPLC-MS/MS with ESI capability) in the positive mode using methods described by Warth et al. (2012). Samples were warmed to 37 °C, centrifuged for 20 min at 2000 x g and defatted. Samples were passed through a coffee filter by gravity flow to remove residual fat, and then 10 mL of the sample was passed through an immunoaffinity column (Alfa WB, Vicam, Milford, MA) at a steady gravity controlled flow rate (approximately 1 mL/min). Columns were then washed twice using 10 mL of double distilled, deionized water (MilliQ 18.2 MΩcm) and eluted with 4 mL of acetonitrile. Samples were evaporated to dryness under constant nitrogen and then re-suspended in 1 mL of 1:1 MeOH water solution and analyzed by LC-MS/MS (Waters H-class UPLC-

MS/MS with ESI capability) in the positive mode for AFM1 (mol. Wt. 328). The mobile phase consisted of an isocratic gradient of 30 % water and 70% acetonitrile, each containing 0.1% formic acid, at a flow rate of 0.325 mL/min. Column temperature and injection volume were 40 °C and 10 µL, respectively. Aflatoxin standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and AF concentrations were determined with instrument software (Empower 2, Waters Corporation, Milford, MA).

Aflatoxin secretion and transfer were calculated. Aflatoxin secreted represents the calculated amount of AFM1 present in the milk and was determined by multiplying AFM1 concentration by milk yield. Aflatoxin transfer represents the amount of AFM1 present in the milk compared to the amount of AFB1 fed daily and was determined by dividing AFM1 secreted by AFB1 intake and multiplying by 100.

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

$$AF\ transfer = \left(\frac{\mu g\ AF\ secretion}{\mu g\ AF\ consumed}\right) \times 100 \qquad 2.2$$

Statistical Analysis

Data were analyzed as a triplicate 5 x 5 Latin square design using SAS® (version 9.4, SAS Institute Inc., Cary, NC). Milk components from each treatment period were represented by milk samples collected on d 4 and 5. Milk yield, milk composition, DMI, feed composition, and feed efficiency were analyzed using the MIXED procedure of SAS, and means were reported LSMEANS (Fisher's Least Significant Differences). A Tukey's test was used to assess differences between treatment means for AFM1

variables. Treatment, DIM, and period were considered independent variables, and milk yield, DMI, AFM1 concentration, and milk composition were dependent variables. Significance was declared when $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.15$. All data were presented as mean ± the largest SEM.

Table 2.1 Ingredient and analyzed chemical composition of diets fed to lactating Holsteins to evaluate the ability of Mycoad clay to mitigate aflatoxin transfer.

	Treatment[1]					
Item	CON	AF	MYC	MYC+AF	SEM[2]	$P <$[3]
Dietary Ingredient (% DM)						
Alfalfa Balage	6.0	6.0	6.0	6.0	-	-
Bermudagrass Balage	2.0	2.0	2.0	2.0	-	-
Corn Silage	39.0	39.0	39.0	39.0	-	-
Bermudagrass hay	1.0	1.0	1.0	1.0	-	-
Whole cotton seed	4.0	4.0	4.0	4.0	-	-
Energy Booster®[4]	1.0	1.0	1.0	1.0	-	-
Concentrate premix[5]	47.0	47.0	47.0	47.0	-	-
Composition						
DM, %	55.8	54.9	55.3	54.5	-	-
Ash, %	8.1	8.2	8.7	8.4	0.21	0.7
CP, %	17.4	17.3	17.1	17.5	0.34	0.63
NDF, %	37.8	39.5	38.5	40.5	2.07	0.81
ADF, %	18.7	19.0	18.6	17.9	0.38	0.4
AF, ppb	0	300	0	300	-	-
Mycoad, g	0	0	50	50	-	-

[1]CON = basal TMR; AF = basal TMR + 300ppb AF; MYCOAD = basal TMR + 50g Mycoad; MYC+AF = basal TMR + 50g Mycoad and 300ppb AF.
[2]Greatest standard error of treatment mean.
[3]Main effect of treatment
[4]Hubbard feeds, Mankato, MN
[5]Contained grain products, plant products, roughage products, forage products, cane molasses, salt, vitamin A acetate, vitamin D3 supplement, vitamin E supplement, zinc oxide, zinc sulfate, manganous oxide, manganous sulfate, copper sulfate, cobalt carbonate, calcium iodate, and sodium selenite (16% Dairy Feed, Ware Milling, Houston, MS)

Table 2.2 Ingredient and analyzed chemical composition of basal diet fed to lactating Holsteins to evaluate the ability of NovaSil Plus clay to mitigate aflatoxin transfer.

Item	Value
Dietary Ingredient (% DM)	
Alfalfa Balage	6.0
Bermudagrass Balage	2.0
Corn Silage	39.0
Bermudagrass hay	1.0
Whole cotton seed	4.0
Energy Booster®[1]	1.0
Concentrate premix[2]	47.0
Composition	
DM, %	56.30
Ash, %	7.54
CP, %	17.22
NDF, %	34.83
ADF, %	18.32

[1]Hubbard feeds, Mankato, MN

[2]Contained grain products, plant products, roughage products, forage products, cane molasses, salt, vitamin A acetate, vitamin D3 supplement, vitamin E supplement, zinc oxide, zinc sulfate, manganous oxide, manganous sulfate, copper sulfate, cobalt carbonate, calcium iodate, and sodium selenite (16% Dairy Feed, Ware Milling, Houston, MS)

CHAPTER III

RESULTS AND DISCUSSION

Experiment One

Feed Composition and Intake

Nutrient composition of TMR treatments was similar, and averaged 55.1% DM, 8,4% Ash, 17.3% CP, 39.1% NDF, and 18.6% ADF ($P > 0.05$; Table 2.1). Dry matter intake, crude protein intake, organic matter intake, NDF intake, and ADF intake were similar among treatments, parity, and DIM ($P > 0.05$; Table 3.1). The similarity of intake among treatments reflects reported results of previous studies (Applebaum et al., 1982; Kutz et al., 2009; Queiroz et al., 2012; Maki et al., 2016a; Maki et al., 2016b; Sulzeberger et al., 2017). While numerous studies have reported the affect of inclusion of AF anf clay in the diets of dairy animals on performance, these studies do not evaluate the effects of parity and DIM as well. However, many studies have evaluated the effects of DIM (Sharma et al., 1990; Friggens et al., 1998; Hagnestam-Nielsen et al., 2009) and parity (Cappio-Borlino et al., 1997; Sevi et al. 2000; Yang et al., 2013) on lactational performance in dairy animals. Davidson et al. (2003) reported greater DMI by multiparous cows compared to primiparous cows, which contrasts the current results. The current study used a greater proportion of primiparous cows compared to that by Davidson et al. (2003), however, and the aforementioned study fed diets of varying protein concentration rather than one ration with a top-dressed AF treatment. Results by

Friggens et al. (1998) also contrast the current results, reporting a decrease of DMI by cows as lactation progressed. However, the study compared cows during wk 8 to 21 of lactation to wk 24 to 37 of lactation, while the current study utilized cows averaging wk 17 and 53 of lactation.

Milk Yield

Milk yield was similar among treatments and averaged 32.6, 36.1, 35.2, and 36.6 ± 1.30 kg/d for cows fed CON, AF, MYCOAD, and MYCOAD+AF diets, respectively ($P > 0.05$; Table 3.2). This was consistent with results from previous studies that reported no difference of milk yield (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017), however contrasted other studies reporting a decrease of milk yield by cows (Applebaum et al., 1982) and ewes (Battacone et al., 2003) consuming AF. Applebaum et al. (1982) reported a decrease of milk yield by cows fed impure AFB1. The study used fewer animals, however, and the study was not performed at one time, but rather when animals were available. Battacone et al. (2003) reported no change of milk yield by ewes consuming 0.767 µg AFB1/kg BW compared to those not consuming AF, however a suppression of production was observed when AFB1 was increased to 1.587 and 3.308 µg AFB1/kg BW. Multiparous animals averaged 36.1 ± 1.64 kg milk/d, and produced more milk than primiparous animals, which averaged 32.5 ± 0.99 kg milk/d ($P < 0.04$; Table 3.2). This was expected, as mammary development is still occurring, and is consistent with previous studies with cows (Yang et al., 2013) and ewes (Cappio-Borlino et al., 1997). In contrast, Sevi et al. (2000) reported no difference of milk yield among parities. Milk yield was also decreased in late-lactation animals compared to mid-lactation animals and averaged 37.6 and 31.0 ± 0.92 kg/d for mid- and late-lactation

animals, respectively ($P < 0.001$; Table 3.2). Numerous studies have also reported reduction losses as lactation progressed (Sharma et al., 1990; Friggens et al., 1998; Hagnestam-Nielsen et al., 2009), and this result was expected because after peak production, milk yield continues to decrease. Therefore, a decrease of milk yield should be reflected in a later stage in lactation, or a greater DIM. The effect of parity and DIM on milk production emphasizes why animals were blocked by these factors. It is important to note that none of the compared studies of parity and DIM were feeding AF diets, but were rather evaluating the affect of parity or DIM on performance.

Feed Efficiency

Feed efficiency was similar across treatments and parity ($P > 0.05$; Table 3.2). Sulzberger et al. (2017) reported a decrease in efficiency when clay was added to AF contaminated diets, but no difference was observed between AF diets containing no clay and control diets containing no AF or clay. This contradicts the current results showing no difference of feed efficiency when clay was added to AF contaminated diets. However, animals in the aforementioned study were administered AFB1, AFB2, AFG1, and AFG2 through a gelatin capsule, which differs from the current study that top-dressed AFB1 onto the TMR. The similarity across parities was unexpected as milk yield was increased in multiparous cows while DMI was not different. Feed efficiency was greater in late-lactation cows, and averaged 0.79 and 0.90 ± 0.06 kg DMI/kg milk for mid- and late-lactation cows, respectively ($P < 0.01$). This reduction in efficiency as DIM increased is expected, as milk yield was reduced while DMI remained similar among DIM.

Milk Composition

Milk Fat

Milk fat was unaffected by treatment or parity ($P > 0.05$; Table 3.2). The effect of treatment on milk fat is similar to numerous studies (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). However, the results differ from those reported by Quieroz et al. (2012). While the authors reported no change of milk fat percent, a reduction of milk fat yield was reported by cows consuming diets contaminated with 75 ppb AFB1 compared to those not administered AF. Results from the current study are also consistent with studies conducted on dairy ewes that reported no change of fat content of milk (Battacone et al., 2003; Battacone et al., 2005), whey, or curds (Battacone et al., 2005) by ewes consuming AF. The similarity among parities contrasts results from previous studies with cows (Yang et al., 2013) and ewes (Cappio-Borlino et al., 1997; Sevi et al., 2000). Cappio-Borlino et al. (1997) and Sevi et al. (2000) reported an increase of milk fat percent in 3rd lactation ewes compared to primiparous ewes, with 2nd lactation ewes being intermediate. Yang et al. (2013) reported an increase in fat percent in multiparous Holstein cow compared to primiparous cows. Yang et al. (2013) also reported an increase in milk fat percent in 2nd and 3rd lactation cows compared to primiparous cows, however there was no difference in 4th lactation cows compared to other parities. The current study did not differentiate between the parities of multiparous animals, however, which may explain the contrasting results of the previously mentioned studies. There was a tendency ($P < 0.08$) for a decrease of milk fat yield as DIM increased. Milk fat yield averaged 1.69 1.73 ± 0.14 kg/d for mid- and late-lactation cows, respectively. Sharma et al. (1990) reported a steady decrease of milk fat from the start of

lactation in Holstein cows. Friggens et al. (1998) reported no change of milk fat yield by cows during wk 8 to 21 of lactation compared to wk 24 to 37 of lactation. This tendency was likely result of decreased milk yield in late-lactation cows compared to mid-lactation cows, as was the result of Sharma et al. (1990). The authors reported a decrease of milk fat percent and milk yield as lactation persisted, resulting in a reduction of milk fat yield.

Milk Protein

Milk protein was unaffected by treatment ($P > 0.05$). This is consistent with results from previous studies, reporting no change of milk protein percent (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017) and yield (Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017) due to the inclusion of AF or clay in the diet. The results from the current study are also consistent with those with ewes consuming AF, reporting no change of protein percent in milk (Battacone et al., 2003; Battacone et al., 2005), cheese, or whey (Battacone et al., 2005). However, Quieroz et al. (2012) reported no difference of protein yield, but a suppression of milk protein percent was observed in cows consuming AFB1 with no clay compared to those consuming AFB1 with clay and those not consuming AF. The authors also reported an increase of protein percent when clay was included at 1% of the diet compared to 0.2%, however control was intermediate. Milk from primiparous cows tended ($P < 0.08$) to contained less protein than milk from multiparous cows and averaged 2.85 and 3.05 ± 0.08% for primiparous and multiparous cows, respectively. Primiparous cows also produced less milk protein than multiparous cows ($P < 0.01$), and milk protein averaged 1.00 and 1.15 ± 0.03 kg/d for primiparous and multiparous cows, respectively. This is consistent with previous results with ewes (Cappio-Borlino et al., 1997; Sevi et al., 2000). Sevi et al.

(2000), reported an increase of milk protein percent in 3rd lactation cows compared to primiparous cows, similar to Cappio-Borlino et al. (1997), reporting an increase of milk protein by 3rd lactation ewes compared to primiparous and 2nd lactation ewes. However, Yang et al. (2013) reported variation in protein percent. The authors reported an increase of milk protein percent by 4th lactation cows compared to 2nd lactation cows, but primiparous and 3rd lactation cows were intermediate of the two. Milk protein percent was unaffected by stage of lactation ($P > 0.05$; Table 3.2). This contrasts a previous study, reporting a decline in protein percent throughout lactation (Sharma et al., 1990). There was a tendency for a decrease of milk protein yield as DIM increased ($P < 0.08$). Milk protein yield averaged 1.11 and 1.04 kg/d for mid- and late-lactation cows, respectively. Friggens et al. (1998) who reported an increase in protein yield wk 24 to 37 compared to wk 8 to 21. Like fat yield, this is likely due to increased milk yield observed by multiparous animals, causing a tendency for an increase in protein yield, as was reported by Sharma et al. (1990).

Lactose

Treatment did not affect lactose percent or yield ($P > 0.05$). This is consistent with results from previous studies with cows (Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017) and ewes (Battacone et al., 2003) consuming AF diets. The consistency of lactose among treatments is also explained by consistency of milk yield among treatments, as lactose drives the production of milk. Milk from primiparous cows contained more lactose than milk from multiparous cows ($P < 0.01$), however lactose yield was unaffected by parity ($P > 0.05$). Lactose percent averaged 4.92 and 4.49 ± 0.11% for primiparous and multiparous cows, respectively. This is similar to previous

studies (Sevi et al., 2000; Lang et al., 2013). Stage of lactation did not affect lactose percent ($P > 0.05$), but lactose yield decreased as DIM increased ($P < 0.01$). Milk lactose yield averaged 1.79 and 1.56 for mid- and late-lactation cows, respectively. The consistency of lactose percent throughout lactation contrasts results reported by Sharma et al. (1990). The authors reported a peak of lactose percent at 2.2 mo followed by a decline throughout lactation, however this combined with the reduction in milk yield as DIM in the current study support the reduction in lactose yield by late-lactation cows. The decrease of lactose yield as DIM increased is consistent with results reported by Friggens et al. (1998).

Milk Solids

Milk solids were unaffected by treatment or parity ($P > 0.05$). This is likely due to the similarities of milk composition among treatments. The similarity among parities is consistent with results by Yang et al. (2013), who did not report a decrease in milk solids percent until the 4th lactation, which was not represented in the current study. Solids percent was unaffected by stage of lactation ($P > 0.05$), however as DIM increased, solids yielded decreased ($P < 0.01$). Milk solids yield averaged 3.25 and 2.89 ± 0.10 kg/d for mid- and late-lactation cows, respectively. This contrasts the results reported on solids non-fat (SNF) by Sharma et al. (1990), displaying a decrease in SNF after the start of the study. Although this analysis did not include fat percent, fat percent also declined throughout lactation (Sharma et al., 1990).

Milk Urea Nitrogen

Milk urea nitrogen was not affected by treatment, parity, or stage of lactation ($P > 0.05$). The similarity among treatments is similar to results from previous studies in cows (Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017) and ewes (Battacone et al., 2005) consuming AF and clay diets. Davidson et al. (2003) also reported similar MUN among parities. However, Jílek et al. (2003) reported an increase in MUN in 3rd and 4th lactation cows compared to primiparous and 2nd lactation cows. Jílek et al. (2003) also reported increasing MUN concentrations throughout the first 5 mo of lactation with little change throughout the rest of lactation, which may be correspond to the current study showing no difference in MUN among mid- and late-lactation cows.

Somatic Cell Count

Somatic cell count was similar across treatments, parity, and DIM ($P > 0.05$). Numerous studies have reported no change of SCC by cows (Kutz et al., 2009; Quieroz et al., 2012; Sulzberger et al., 2017) and ewes (Battacone et al., 2003; Battacone et al., 2005) consuming AF and clay diets. Sevi et al. (2000) reported no chance in SCC among parity on lactating ewes. In contrast, Sheldrake et al. (1983) and Yang et al. (2013) reported increases in SCC as parity increased. Although Sheldrake et al., (1983) reported an increase in SSC with increasing number of lactations, the authors attributed this to infection rather than a direct result from parity, as there was little change in SCC in quarters free from infection. Sheldrake et al. (1983) also reported an increase in SCC as DIM increased, however the authors again attributed this to disease rather than a direct result from increasing DIM.

Aflatoxin M_1

Concentration of Aflatoxin M_1

Aflatoxin M_1 concentration was unaffected by parity or DIM, but averaged 0.25, 2.27, 0.16, and 0.83 ± 0.30 ppb for CON, AF, MYCOAD, and MYCOAD+AF cows, respectively ($P < 0.001$; Table 3.3). Daily averages of AFM1 by treatment are displayed in Figure 3.1. Milk from cows consuming AF diets contained the greatest concentration of AFM1, and all other diets were similar. Inclusion of Mycoad in the diet resulted in a 53.4% reduction in AFM1. The reduction in AFM1 concentration in cows administered a clay adsorbent is consistent with previous studies (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). The results from the current study contrast those reported by Queiroz et al. (2012), in which there was no difference in AFM1 concentration between cows fed AF diets with the addition of 0.2% montmorillonite clay, compared to those not consuming clay. The authors did report a decrease in AFM1 concentration when the clay was increased from 0.2 to 1.0% DMI. Although all concentrations of AFM1 are greater than the legal action limit of 0.5 ppb, AF was included in the diet at 300 ppb, which is much greater than the action limit in lactating feeds (20 ppb). Milk from cows consuming CON and MYCOAD diets contained the least amount of AFM1, which was to be expected as no AFB1 was added to the feed. However, AFM1 was still observed in both CON and MYCOAD diets. Contamination of AFM1 in milk from cows that were not purposefully contaminated with AF has been reported in other studies (Maki et al., 2016a; Maki et al., 2016b).

Secretion of Aflatoxin M_1

Secretion of AFM1 was unaffected by parity or DIM, but averaged 5.53, 80.67, 2.65, and 32.61 ± 10.9 µg for CON, AF, MYCOAD, and AF+MYCOAD cows, respectively ($P < 0.001$; Table 3.3). Cows consuming AF diets secreted the greatest amount of AFM1, followed by cows consuming MYCOAD+AF diets. Cows consuming MYCOAD diets secreted the least amount of AFM1, and cows consuming CON diets were intermediate between MYCOAD+AF and CON cows. The reduction in AFM1 secretion following the dietary addition of a clay adsorbent is represented in numerous studies (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). Contrasting results were reported by Quieroz et al. (2012), in which no difference was observed between AF contaminated diets containing no clay adsorbent and those in which a clay adsorbent was administered. However, a reduction was observed when the concentration of clay in the diet was increased to 1.0% compared to 0.2% DMI.

Transfer of Aflatoxin M_1

Transfer of AF was reduced from 1.15 to 0.42 ± 0.25% when cows consumed MYCOAD+AF diets compared to AF diets, respectively ($P < 0.045$; Table 3.3). Inclusion of Mycoad resulted in a 63.5% reduction in transfer. This is consistent with results from previous studies reporting a reduction in AFM1 transfer percent with the addition of a clay adsorbent to AF contaminated feed (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). Like AFM1 secretion, Quieroz et al. (2012) reported no difference in AFM1 transfer between AF contaminated diets with a clay adsorbent, and diets containing no clay. However, a reduction in AFM1 transfer was observed when the concentration of clay in the diet was increased from 0.2% to 1.0%

DMI. Parity did not affect AF transfer ($P > 0.05$), but there was a tendency AF transfer to be greater in mid-lactation cows compared to late-lactation cows, and averaged 1.07 and 0.51 ± 0.27% for mid- and late-lactation cows, respectively ($P < 0.06$).

Experiment Two

Intake

Dry matter intake, crude protein intake, organic matter intake, NDF intake, and ADF intake were similar among treatments ($P > 0.05$; Table 3.4). Dry matter intake averaged 32.55, 33.34, 33.71, 33.48, and 34.46 ± 0.81 kg/d for treatments CON, NSPC, AFC, NSP-0.125%, and NSP-0.25%, respectively. The similar DMI among treatments is in accordance with numerous studies feeding AFB1 and adsorbents (Applebaum et al., 1982; Kutz et al., 2009; Queiroz et al., 2012; Maki et al., 2016a, Maki et al., 2016b; Sulzberger et al., 2017).

Milk Yield

Milk yield was similar among treatments and averaged 36.96, 37.12, 36.45, 36.27, and 36.18 ± 0.75 kg/d for cows consuming CON, NSPC, AFC, NSP-0.125%, and NSP-0.25% diets, respectively ($P < 0.90$; Table 3.5). Numerous studies have shown no change in milk production following the consumption of AFB1 or adsorbent clay (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). However, other studies have reported a suppression of milk yield in cows (Applebaum et al., 1982) and ewes (Battacone et al., 2003) consuming AF. Battacone et al. (2003) reported no change in milk yield in ewes consuming 32 µg AFB1/d compared to those not consuming AF, however a suppression in production was observed when AFB1 was increased to 64 or

128 µg/d. Applebaum et al. (1982) reported no change in milk yield in cows consuming diets contaminated with pure AFB1, however there was a decrease in milk yield in animals consuming impure AFB1 compared to control animals averaging 2.4 kg/d.

Feed Efficiency

Feed efficiency was unaffected by treatment ($P > 0.05$; Table 3.5), which contrasts research by Sulzberger et al. (2017) where a decrease in feed efficiency (kg milk/kg DMI) was observed in cows administered 100 ppb AFB1 and a clay adsorbent compared to cows administered AF with no clay. Although the current results contrast previous research, the similarity in efficiency among treatments was expected as DMI and milk yield were also similar among treatments.

Milk Composition

Milk Fat

Milk fat yield was unaffected by treatment ($P > 0.05$), and percent averaged 4.22, 4.49, 4.38, 4.75, and 4.61 ± 0.08% for animals consuming CON, NSPC, AFC, NSP-0.125%, and NSP-0.25% diets, respectively ($P < 0.01$; Table 3.5). Animals consuming NSP-0.125% diets produced the greatest percentage of milk fat followed by NSPC cows with NSP-0.25% cows being an intermediary. Cows consuming CON diets produced the least amount of milk fat with AFC cows intermediary between CON and NSPC cows. These results differ from previous studies that reported no differences in milk fat percent (Kutz et al., 2009; Maki et al., 2012a; Maki et al., 2016b; Sulzeberger, 2017). Queiroz et al. (2012) reported no change in milk fat percent, but a suppression in milk fat yield was reported in cows consuming diets contaminated with 75 ppb AFB1 compared to cows not

administered AF. The results from this study also agree with studies conducted on dairy ewes that reported no change in milk fat in ewes consuming AF in milk (Battacone et al., 2003; Battacone et al., 2005), whey, or curds (Battacome et al., 2005).

Milk Protein

Milk protein yield was similar across treatments, and percent averaged 2.93, 2.96, 2.98, 2.92, and 3.02 ± 0.02% for cows consuming CON, NSPC, AFC, NSP-0.125%, and NSP-0.25% diets, respectively ($P < 0.01$; Table 3.5). Milk from cows fed NSP-0.25% diets contained the greatest concentration of protein compared to that of cows fed NSP-0.125% diets. Milk protein from AFC cows was similar to that all other treatments except NSP-0.125% cows. Milk protein from NSPC cows was similar to milk protein percent from all other treatments, and milk protein from CON cows was similar to all treatments except NSP-0.25 cows. Like the results of milk fat percent observed, these results are consistent with studies conducted using cows (Kutz et al., 2009; Maki et al., 2012a; Maki et al., 2016b) and ewes (Battacone et al., 2003; Battacone et al., 2005; Sulzeberger et al., 2017), with the exception of the results presented by Queiroz et al. (2012). Queiroz et al. (2012) reported a decrease in milk protein percent in cows fed 75 ppb AFB1 compared to control animals that received no AF.

Lactose

Lactose percent and yield were unaffected by treatment ($P > 0.05$; Table 3.5), which is consistent with previous studies in cows (Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017) and ewes (Battacone et al., 2003) consuming AF contaminated diets.

Milk Solids

Solids yield was unaffected, but there was a tendency for treatment effect in milk solids content ($P < 0.07$; Table 3.5). Milk from cows consuming AF diets tended to contain more solids than that of cows consuming CON diets, with NSP cows being intermediate. Milk from cows consuming NSP-0.125% diets tended to be similar to that of cows consuming CON and NSP diets, and milk solids content of cows consuming NSP-0.25% diets tended to be similar to that of AF and NPS-0.125% cows.

Milk Urea Nitrogen

Milk urea nitrogen was unaffected by treatment ($P > 0.05$; Table 3.5). This was consistent with results from previous studies in cows (Maki et la., 2016a; Maki et al., 2016b; Sulzberger et al., 2017) and ewes (Battacone et al., 2005) consuming AF and clay diets.

Somatic Cell Count

A tendency was observed for an increase in SCC ($P < 0.06$; Table 3.5). This is possibly due to cow variation and normal incidence of disease in the herd. Previous studies reported no change in SCC of cows (Queiroz et al., 2012; Sulzberger et al., 2017) and ewes (Battacone et al., 2003; Battacone et al., 2005) consuming AF contaminated feed, although many studies did not report SCC (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b). Animals consuming NSP-0.25% diets tended to have increased SCC, however NSPC cows were similar to CON, and AFC cows were similar across all treatments. The tendency for this increase does not appear to be attributed to AF or inclusion of NSP in the diets of lactating cows.

Body Condition Score

Body condition score was unaffected by treatment ($P > 0.05$; Table 3.5), which is consistent with previous studies feeding diets containing AF and clay adsorbents (Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017).

Aflatoxin M_1

Concentration of Aflatoxin M_1

Aflatoxin M_1 concentration averaged 0.09, 0.03, 0.75, 0.62, and 0.59 ± 0.02 ppb for CON, NSPC, AFC, NSP+0.125%, NSP+0.25% diets, respectively ($P < 0.001$; Table 3.6). A decrease in AFM1 concentration in the milk of cows consuming contaminated diets due to administration of clay adsorbents is well documented in research (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). This decrease was consistent with the current results, which showed that milk from cows consuming AFC diets contained the greatest concentration of AFM1. Aflatoxin M_1 was reduced by 17.3% with the inclusion of NSP at 0.125% DMI and by 21.3% when NSP was included at 0.25% DMI, although both diets were similar with respect to AFM1 concentration. This similarity contrasts previous results in which a dose dependent response was observed (Maki et al., 20116a; Maki et al., 2016b). The reduction in AFM1 with such minute inclusions of NSP contrasts results reported by Queiroz et al. (2012), which showed no reduction in AFM1 concentration with the addition of a montmorillonite clay adsorbent at 0.2% DMI. However, a reduction was observed when the clay was increased to 1% DMI. Milk from cows consuming CON and NSPC diets contained the least amount of AFM1, which is expected as no additional AF was added to the feed. However, AFM1 was still observed in both CON and NSPC diets. Contamination of AF in milk from cows that

were not purposefully contaminated with AF has been reported in other studies (Maki et al., 2016a; Maki et al., 2016b). The presence of naturally occurring AFM1 reiterates the importance of evaluating methods to mitigate AF transfer.

Secretion of Aflatoxin M_1

Secretion of AFM1 averaged 3.27, 1.10, 29.4, 24.7, and 23.9 ± 1.47 µg for CON, NSPC, AFC, NSP-0.125%, and NSP-0.25% cows, respectively ($P < 0.001$; Table 3.6). Cows consuming AFC diets secreted the greatest amount AFM1 followed by NSP-0.125% and NSP-0.25% diets, which were not different from each other. Cows consuming CON and NSP diets secreted the least amount of AFM1. The reduction in secretion of AFM1 is in agreement previous research (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017). In previous studies, the addition of NSP in the AF contaminated diets reduced AFM1 secretion at 0.5, 0.6, 1.0, and 1.21% DMI compared to diets containing no AF (Maki et al., 2016a; Maki et al., 2016b). Contrasting results were reported by Quieroz et al. (2012), in which no difference was observed between AF contaminated diets containing no clay adsorbent and those in which a clay adsorbent was administered. However, a reduction was observed when the concentration of clay in the diet was increased to 1.0% compared to 0.2% DMI.

Transfer of Aflatoxin M_1

Transfer of AFM1 averaged 1.78, 1.49, and 1.46 for AFC, NSP-0.125, and NSP-0.25% cows, respectively, and a reduction in transfer was observed in NSP-0.125% and NPS-0.25% cows compared to AFC diets ($P < 0.01$; Table 3.6). No difference in AFM1 transfer was observed between NSP-0.125% and NSP-0.25% cows. A reduction in AFM1

transfer following the inclusion of a clay adsorbent is well documented in research (Kutz et al., 2009; Maki et al., 2016a; Maki et al., 2016b; Sulzberger et al., 2017) and has been observed by cows consuming NSP in greater concentrations than the current study (Maki et al., 2016a; Maki et al., 2016b). Similar to AFM1 secretion, Quieroz et al. (2012) reported no difference in AFM1 transfer between AF contaminated diets with 0.2% of a montmorillonite clay adsorbent, and diets containing no clay. However, a reduction in AFM1 transfer was observed when the concentration of clay in the diet was increased from 0.2% to 1.0% DMI. Previous studies feeding NSP reported a dose-dependent response to reduction in AFM1 transfer (Maki et al., 2016a; Maki et al., 2016b), which was not observed in the current study.

Table 3.1 Effect of dietary addition of MYCOAD[1] on intake of dairy cows consuming a known concentration of aflatoxin (AF)

	Treatment[3]					Parity			DIM[4]			P<[5]		
Item[2], kg	CON	AF	MYCOAD	MYCOAD+ AF	SE[6]	1	2+	SE[6]	125	375	SE[6]	Trt	Parity	DIM
DMI	32.2	28.0	31.9	28.8	2.92	30.0	30.4	2.40	29.3	31.1	1.96	0.58	0.90	0.47
CPI	5.36	4.98	5.57	5.10	0.42	5.05	5.44	0.33	5.07	5.42	0.28	0.62	0.36	0.31
OMI	28.3	26.3	29.4	27.0	2.27	27.0	28.5	1.77	26.6	28.8	1.53	0.63	0.50	0.26
NDFI	11.7	11.0	12.4	11.2	0.96	11.2	12.0	0.75	11.1	12.1	0.65	0.59	0.41	0.25
ADFI	5.61	5.19	5.79	5.33	0.44	5.40	5.56	0.35	5.26	5.70	0.30	0.64	0.73	0.23

[1]MYCOAD is a bentonite clay with greater than 80 % smectite content (Special Nutrients, Miami, FL).

[2]DMI = dry matter intake; CPI = crude protein intake; OMI = organic matter intake; NDFI = neutral detergent fiber; ADFI = acid detergent fiber intake

[3]**CON** = basal TMR; **AF** = basal TMR + 300 ppb AF; **MYCOAD** = basal TMR + 50 g MYCOAD; **MYCOAD+AF** = basal TMR + 300 ppb AF + 50 g MYCOAD

[4]DIM = days in milk; mid-lactation animals averaged 125 DIM; late-lactation animals averaged 375 DIM

[5]Main effect of treatment

[6]Greatest standard error of treatment mean

Table 3.2 Effect of dietary addition of Mycoad[1] on performance of dairy cows consuming a known concentration of aflatoxin (AF)

	Treatment[3]					Parity			DIM[4]			P<[5]		
Item[2]	CON	AF	MYCOAD	MYCOAD+AF	SE[6]	1	2+	SE [6]	125	375	SE [6]	Trt	Parity	DIM
MY, kg/d	32.6	36.1	35.2	36.6	1.383	33.3	36.9	0.99	125	375	SEM	0.19	0.01	0.001
FE	0.92	0.82	0.93	0.82	0.080	0.90	0.88	0.074	37.6	32.6	0.92	0.28	0.78	0.01
Fat, kg	1.48	1.46	1.38	1.80	0.193	1.46	1.60	0.151	0.79	0.90	0.064	0.39	0.49	0.08
Fat, %	4.83	4.67	4.88	5.99	0.652	5.05	5.13	0.535	1.69	1.37	0.130	0.36	0.91	0.81
Protein, kg	0.99	1.13	1.09	1.09	0.065	1.00	1.15	0.051	5.03	5.16	0.437	0.25	0.01	0.08
Protein, %	2.75	3.11	2.99	2.96	0.101	2.85	3.05	0.083	1.11	1.04	0.044	0.11	0.08	0.18
Lactose, kg	1.56	1.68	1.65	1.80	0.092	1.65	1.69	0.072	2.89	3.01	0.068	0.29	0.60	0.01
Lactose, %	4.87	4.57	4.62	4.77	0.131	4.92	4.49	0.107	1.79	1.56	0.062	0.25	0.01	0.65
Solids, kg	2.85	3.13	3.06	3.24	0.168	2.97	3.17	0.131	4.73	4.68	0.088	0.31	0.16	0.01
Solids, %	8.60	8.56	8.50	8.66	0.213	8.75	8.41	0.174	3.25	2.89	0.113	0.92	0.17	0.77
MUN, mg/dL	13.5	10.9	10.3	10.5	1.61	12.0	10.5	1.32	8.55	8.61	0.142	0.44	0.41	0.83
SCC, x10^3	123	155	160	189	103.3	75	238	84.7	11.2	11.5	1.08	0.98	0.17	0.32

[1]Mycoad is a bentonite clay with greater than 80% smectite content (Special Nutrients, Miami, FL).
[2]MY = milk yield; FE = kg DMI/kg milk; SCC = somatic cell count
[3]CON = basal TMR; AF = basal TMR + 300 ppb AF; MYCOAD = basal TMR + 50 g MYCOAD; MYCOAD+AF = basal TMR + 300
ppb AF + 50 g MYCOAD
[4]DIM = days in milk; mid-lactation animals averaged 125 DIM; late-lactation animals averaged 375 DIM
[5]Main effect of treatment
[6]Greatest standard error of treatment mean

Table 3.3 Effect of dietary addition of Mycoad[1] on performance of dairy cows consuming a known concentration of aflatoxin (AF)

	Treatment[3]					Parity			DIM[4]			P<[5]		
Item[2]	CON	AF	MYCOAD	MYCOAD+AF	SE[6]	1	2+	SE [6]	125	375	SE [6]	Trt	Parity	DIM
AFM1, ppb	0.25[a]	2.27[b]	0.16[a]	0.83[a]	0.300	0.94	0.81	0.24	1.04	0.71	0.180	0.001	0.65	0.20
Secretion, µg[7]	5.53[a]	80.67[c]	2.65[a]	32.61[b]	10.900	30.2	30.6	8.27	36.47	23.38	7.699	0.001	0.97	0.20
Transfer, %[8]	N/A	1.15	N/A	0.42	0.254	0.78	0.80	0.32	1.07	0.51	0.268	0.045	0.96	0.06

[1]Mycoad is a bentonite clay with greater than 80% smectite content (Special Nutrients, Miami, FL).
[2]AFM1 = Aflatoxin M_1
[3]CON = basal TMR; AF = basal TMR + 300 ppb AF; MYCOAD = basal TMR + 50 g MYCOAD; MYCOAD+AF = basal TMR + 300
ppb AF + 50 g MYCOAD; means in the same row with different superscripts differ.
[4]DIM = days in milk; mid-lactation animals averaged 125 DIM; late-lactation animals averaged 375 DIM
[5]Main effect of treatment
[6]Greatest standard error of treatment mean
[7]AFM1 concentration multiplied by milk yield based on milk production day of collection
[8]AFM1 secretion divided by AF consumption * 100

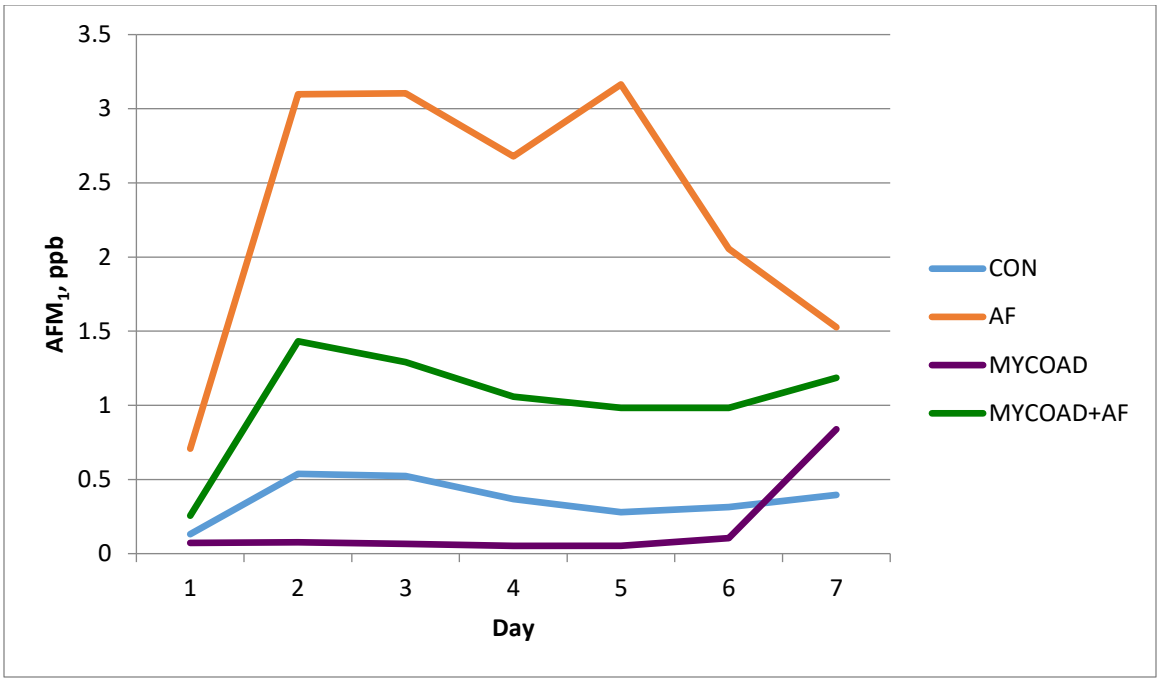

Figure 3.1 Daily concentration of AFM1 in cows fed either 0 or 300 ppb AFB1 and 0 or 50 g Mycoad clay.

CON = 0 ppb AFB1 + 0 g Mycoad, AF = 300 ppb AFB1 + 0 g Mycoad, MYCOAD = 0 ppb AFB1 + 50 g Mycoad, MYCOAD+AF = 300 ppb AFB1 + 50 g Mycoad.

Table 3.4 Effect of dietary addition of NovaSil Plus[1] on intake of dairy cows consuming a known concentration of aflatoxin (AF)

	Treatment[2]						
Item[3]	CON	NSPC	AFC	NSP-0.125%	NSP-0.25%	SEM[4]	P<[5]
DMI, kg/d	32.6	33.3	33.7	33.5	34.5	0.81	0.57
CPI, kg/d	5.63	5.69	5.69	5.74	5.80	0.25	0.90
OMI, kg/d	16.00	16.33	16.50	16.38	16.89	0.70	0.57
NDFI, kg/d	10.92	11.26	11.32	11.37	11.43	0.52	0.77
ADFI, kg/d	5.7	5.91	6.0	6.0	6.1	0.27	0.56

[1]NovaSil Plus (BASF Corp., Ludwigshaven, Germany) is a calcium montmorillonite clay.
[2]CON = basal TMR; AFC = basal TMR + 50ppb AF; NSPC = basal TMR + 0.5% estimated DMI clay; NSP-0.125% = basal TMR + 50ppb AF + 0.125% estimated DMI clay; NSP-0.25% = basal TMR + 50ppb AF + 0.25% estimated DMI clay.
[3]DMI = dry matter intake; CPI = crude protein intake; OMI = organic matter intake; NDFI = neutral detergent fiber intake; ADFI = acid detergent fiber intake
[4]Greatest standard error of treatment mean.
[5]Main effect of treatment

Table 3.5 Effect of dietary addition of NovaSil Plus[1] on performance of dairy cows consuming a known concentration of aflatoxin (AF)

	Treatment[2]						
Item[3]	CON	NPSC	AFC	NSP-0.125%	NSP-0.25%	SEM[4]	P<[5]
MY, kg/d	36.7	37.1	36.5	36.3	36.2	0.75	0.91
FE	0.91	0.95	0.92	0.95	0.97	0.027	0.55
Fat, kg	1.55	1.67	1.61	1.71	1.67	0.050	0.13
Fat, %	4.22[a]	4.50[b]	4.38[a,b]	4.75[c]	4.61[b,c]	0.086	< 0.01
Lactose, kg	1.77	1.80	1.78	1.77	1.74	0.038	0.90
Lactose, %	4.84	4.86	4.89	4.87	4.83	0.024	0.37
Protein, kg	1.08	1.10	1.08	1.05	1.09	0.023	0.57
Protein, %	2.93[a,b]	2.96[a,c]	2.98[b,c]	2.92[a]	3.02[c]	0.022	< 0.01
Solids, kg	3.19	3.23	3.20	3.17	3.16	0.067	0.95
Solids, %	8.69	8.74	8.80	8.72	8.76	0.029	0.07
SCC, x10^3	143	155	217	188	340	53.8	0.06
BCS	2.97	3.00	2.97	2.92	2.95	0.079	0.96

[1]NovaSil Plus (BASF Corp., Ludwigshaven, Germany) is a calcium montmorillonite clay.
[2]CON = basal TMR; AFC = basal TMR + 50ppb AF; NSPC = basal TMR + 0.5% estimated DMI clay; NSP-0.125% = basal TMR + 50 ppb AF + 0.125% estimated DMI clay; NSP-0.25% = basal TMR + 50 ppb AF + 0.25% estimated DMI clay; means in the same row with different superscripts differ.
[3]MY = milk yield; FE = kg DMI/kg milk; BCS = body condition score
[4]Greatest standard error of treatment mean.
[5]Main effect of treatment

Table 3.6 Effect of dietary addition of NovaSil Plus[1] on aflatoxin M_1 (AFM1) content in milk from dairy cows consuming a known concentration of aflatoxin (AF)

	Treatment[2]						
	CON	NSPC	AFC	NSP-0.125%	NSP-0.25%	SEM[3]	*P*<[4]
AFM_1, ppb	0.09[c]	0.03[c]	0.75[a]	0.62[b]	0.59[b]	0.025	0.001
Secretion, µg/d[5]	3.27[c]	1.10[c]	29.4[a]	24.7[b]	23.9[b]	1.470	0.001
Transfer, %[6]	N/A	N/A	1.78[a]	1.49[b]	1.46[b]	0.081	0.01

[1]NovaSil Plus (BASF Corp., Ludwigshaven, Germany) is a calcium montmorillonite clay
[2]CON = basal TMR; NSPC = basal TMR + 125 g of clay; AFC = basal TMR + 50 ppb AF; NSP-0.125% = basal TMR + 32 g of clay + 50 ppb AF; NSP-0.25% = basal TMR + 60 g of clay + 50 ppb AF; means in the same row with different superscripts differ.
[3]Greatest standard error of treatment mean is shown.
[4]Main effect of treatment.
[5] AFM1 concentration multiplied by milk yield based on milk production day of collection
[6] AFM1 secretion divided by AF consumption * 100

CHAPTER IV

CONCLUSION

Both studies demonstrated the efficacy of clay adsorbents at reducing AFM1 transfer into milk of dairy cows. Both Mycoad and NSP were included at minimal concentrations, 0.17% Mycoad and 0.125 or 0.25% NSP. The 0.17% inclusion of Mycoad resulted in a 63.4% reduction in AFM1 concentration and a 63.5% reduction in transfer when cows consumed diets contaminated with 300 ppb AF, 15 times the legal action limit of 20 ppb. Although AFM1 concentrations were not reduced below the action limit of 0.5 ppb, if the feed contamination were reduced further, the milk concentration would most likely fall below that limit. Adding NSP, both 0.125 and 0.25%, reduced the concentration of AFM1 an average of 19.3%, and transfer was reduced an average of 17.1%. Although both concentrations of NSP reduced AFM1 concentration and transfer, there was no elevated reduction when the inclusion of NSP increased from 0.125% to 0.25%. Both clays reduced AFM1 without negatively altering intake, milk yield, or milk composition, indicating that Mycoad and NSP can be included in dairy rations without compromising production. NovaSil Plus has previously been tested at greater concentrations, and the current results will aid in determining the appropriate dosage needed to decrease AFM1 below allowable concentrations. The importance of evaluating AF mitigation techniques was proven by the presence of AFM1 in control diets in both studies, indicating naturally occuring AF in the TMR on the farm. If further research

supports the current studies and more information on proper dosage is obtained through this research, the inclusion of clay adsorbents may be beneficial to dairy producers that are challenged with AF contamination.

The challenges associated with AF contamination are numerous, and advancements in on farm strategies to manage AF are necessary. Discovering the source of AF may present a challenge to producers as testing feed for contamination is not always accurate. Due to the uneven distribution of AF in a feed source, a sample collected may easily overestimate or underestimate the extent of AF in the feed. Additionally, once contamination is observed in the milk, it may still be days before the results of a feed analysis are reported back to the producer. A quick and reliable on farm test for feed contamination that reports concentration of AF would be beneficial to producers, however the challenge of obtaining an accurate sample will still be present. Once AF is present in the milk, strategies to safely remove the toxin without compromising quality would also prevent the cost of discarding milk. Even if successful techniques are presented, consumer opinion may pose a challenge for mitigation techniques of this manner. Because of this and the difficulty of such strategies, preventing AF from transfering into the milk may be the more viable option. Clay adsorbents may be a practical on farm solution to managening AF in dairy herds.

Additionally, clay adsorbents may potentially be used in humans. Aflatoxicosis is not limited to livestock production, and young children are particularly susceptible. If proven safe for human consumption, clay adsorbents may also be used to mitigate AF in developing countries commonly affected by aflatoxicosis.

Overall, results from the current studies indicate that Ca montmorillonite and bentonite clays, specifically NSP and Mycoad, respectively, may be effective and practical additives for dairy producers aiming to reduce AF in milk. However, the challenges associated with AF contamination on dairy farms are still numerous, and further research is needed to develop safe and effective strategies to mitigate AF in dairy herds.

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APPENDIX A

DRY MATTER INTAKE OF COWS FED A KNOWN CONCENTRATION OF AFLATOXIN IN EXPERIMENT ONE

Table A.1 Daily dry matter intake (DMI) of cows fed a known concentration of aflatoxin (AF) and Mycoad clay[1] during experiment one.

Cow ID	Treatment[2]	Day	DMI, kg
306	MYCOAD	1	28.79
309	CON	1	18.88
312	AF	1	24.71
313	MYCOAD+AF	1	27.41
318	CON	1	27.29
330	AF	1	29.90
334	AF	1	22.05
336	MYCOAD+AF	1	33.06
340	MYCOAD	1	21.02
827	CON	1	19.44
845	MYCOAD	1	32.76
859	MYCOAD	1	25.49
862	MYCOAD+AF	1	25.02
864	MYCOAD+AF	1	32.78
895	AF	1	27.05
927	CON	1	19.23
941	CON	1	31.33
970	AF	1	33.79
971	MYCOAD	1	30.47
978	MYCOAD	1	28.40
992	AF	1	34.94
994	MYCOAD+AF	1	28.83
995	AF	1	33.63
996	CON	1	3.59
306	MYCOAD	2	28.94
309	CON	2	22.08
312	AF	2	30.43
313	MYCOAD+AF	2	26.03
318	CON	2	26.11
330	AF	2	31.96
334	AF	2	26.12
336	MYCOAD+AF	2	30.29
340	MYCOAD	2	29.54

Table A.1 (Continued)

827	CON	2	33.32
845	MYCOAD	2	29.36
859	MYCOAD	2	26.03
862	MYCOAD+AF	2	32.69
864	MYCOAD+AF	2	29.16
895	MYCOAD+AF	2	29.41
927	CON	2	18.55
941	CON	2	29.61
970	AF	2	34.99
971	MYCOAD	2	33.99
978	MYCOAD	2	29.17
992	AF	2	35.04
994	MYCOAD+AF	2	27.42
995	AF	2	34.41
996	CON	2	17.81
306	MYCOAD	3	29.98
309	CON	3	32.07
312	AF	3	30.82
313	MYCOAD+AF	3	31.83
318	CON	3	27.78
330	AF	3	31.05
334	AF	3	23.03
336	MYCOAD+AF	3	32.36
340	MYCOAD	3	31.11
827	CON	3	35.39
845	MYCOAD	3	35.19
859	MYCOAD	3	31.08
862	MYCOAD+AF	3	34.45
864	MYCOAD+AF	3	34.17
895	MYCOAD+AF	3	35.01
927	CON	3	20.99
941	CON	3	31.96
970	AF	3	35.08
971	MYCOAD	3	34.89
978	MYCOAD	3	31.11

Table A.1 (Continued)

992	AF	3	35.01
994	MYCOAD+AF	3	33.37
995	AF	3	34.95
996	CON	3	0.49
306	MYCOAD	4	31.95
309	CON	4	34.45
312	AF	4	30.86
313	MYCOAD+AF	4	32.48
318	CON	4	30.64
330	AF	4	32.87
334	AF	4	27.50
336	MYCOAD+AF	4	34.74
340	MYCOAD	4	29.19
827	CON	4	35.72
845	MYCOAD	4	35.33
859	MYCOAD	4	31.58
862	MYCOAD+AF	4	35.02
864	MYCOAD+AF	4	35.08
895	MYCOAD+AF	4	33.57
927	CON	4	19.25
941	CON	4	34.47
970	AF	4	34.89
971	MYCOAD	4	35.38
978	MYCOAD	4	29.42
992	AF	4	35.10
994	MYCOAD+AF	4	34.98
995	AF	4	34.89
996	CON	4	4.53
306	MYCOAD	5	28.14
309	CON	5	30.78
312	AF	5	30.45
313	MYCOAD+AF	5	34.76
318	CON	5	33.64
330	AF	5	30.55
334	AF	5	30.21

Table A.1 (Continued)

336	MYCOAD+AF	5	34.17
340	MYCOAD	5	33.47
827	CON	5	35.06
845	MYCOAD	5	33.84
859	MYCOAD	5	28.59
862	MYCOAD+AF	5	33.60
864	MYCOAD+AF	5	34.55
895	MYCOAD+AF	5	32.08
927	CON	5	30.79
941	CON	5	31.46
970	AF	5	34.34
971	MYCOAD	5	35.43
978	MYCOAD	5	30.45
992	AF	5	35.08
994	MYCOAD+AF	5	34.67
995	AF	5	34.11
996	CON	5	10.38
306	MYCOAD	6	29.93
309	CON	6	34.06
312	AF	6	26.12
313	MYCOAD+AF	6	34.37
318	CON	6	27.98
330	AF	6	33.68
334	AF	6	21.06
336	MYCOAD+AF	6	32.52
340	MYCOAD	6	31.45
827	CON	6	35.49
845	MYCOAD	6	33.21
859	MYCOAD	6	29.90
862	MYCOAD+AF	6	26.90
864	MYCOAD+AF	6	33.67
895	MYCOAD+AF	6	32.10
927	CON	6	23.55
941	CON	6	32.68
970	AF	6	34.05

Table A.1 (Continued)

971	MYCOAD	6	28.93
978	MYCOAD	6	28.59
992	AF	6	35.03
994	MYCOAD+AF	6	30.55
995	AF	6	34.79
996	CON	6	-3.64
306	MYCOAD	7	25.23
309	CON	7	35.56
312	AF	7	30.85
313	MYCOAD+AF	7	32.16
318	CON	7	35.00
330	AF	7	33.42
334	AF	7	33.55
336	MYCOAD+AF	7	30.51
340	MYCOAD	7	27.85
827	CON	7	35.45
845	MYCOAD	7	34.86
859	MYCOAD	7	31.50
862	MYCOAD+AF	7	34.98
864	MYCOAD+AF	7	34.69
895	MYCOAD+AF	7	31.88
927	CON	7	29.22
941	CON	7	34.43
970	AF	7	34.93
971	MYCOAD	7	35.23
978	MYCOAD	7	32.50
992	AF	7	34.98
994	MYCOAD+AF	7	34.61
995	AF	7	34.98
996	CON	7	21.39

[1]Bentonite clay with > 80% smectite content (Special Nutrients, Miami, FL
[2]CON = 0 ppb AFB_1 + 0 g Mycoad, AF = 300 ppb AFB_1 + 0 g Mycoad, MYCOAD = 0 ppb AFB_1 + 50 g Mycoad clay, MYCOAD+AF = 300 ppb AFB_1 + 50 g Mycoad

APPENDIX B

DRY MATTER INTAKE OF COWS FED A KNOWN CONCENTRATION OF AFLATOXIN IN EXPERIMENT TWO

Table B.1 Daily dry matter intake (DMI) of cows fed a known concentration of aflatoxin (AF) and NovaSil Plus[1] during experiment two.

Cow ID	Period	Treatment[2]	Day	DMI, kg
306	1	CON	1	30.24
309	1	CON	1	32.19
312	1	AFC	1	32.05
313	1	AFC	1	32.90
318	1	AFC	1	26.17
326	1	NSP-0.125%	1	22.86
330	1	NSP	1	32.15
334	1	NSP-0.25%	1	29.64
336	1	NSPC	1	30.47
340	1	NSPC	1	25.47
845	1	NSP-0.25%	1	33.00
862	1	NSP-0.25%	1	38.23
864	1	NSP-0.125%	1	43.43
895	1	CON	1	33.83
941	1	NSP-0.125%	1	32.36
306	1	CON	2	28.99
309	1	CON	2	32.29
312	1	AFC	2	31.10
313	1	AFC	2	32.86
318	1	AFC	2	28.21
326	1	NSP-0.125%	2	27.91
330	1	NSPC	2	34.38
334	1	NSP-0.25%	2	29.85
336	1	NSPC	2	30.82
340	1	NSPC	2	23.46
845	1	NSP-0.25%	2	32.58
862	1	NSP-0.25%	2	38.53
864	1	NSP-0.125%	2	44.08
895	1	CON	2	31.56
941	1	NSP-0.125%	2	31.46
306	1	CON	3	29.78
309	1	CON	3	32.60
312	1	AFC	3	31.84
313	1	AFC	3	30.01
318	1	AFC	3	22.62
326	1	NSP-0.125%	3	28.68

Table B.1 (Continued)

330	1	NSPC	3	32.84
334	1	NSP-0.25%	3	28.33
336	1	NSPC	3	32.36
340	1	NSPC	3	24.62
845	1	NSP-0.25%	3	34.81
862	1	NSP-0.25%	3	36.77
864	1	NSP-0.125%	3	46.27
895	1	CON	3	12.69
941	1	NSP-0.125%	3	32.39
306	1	CON	4	33.29
309	1	CON	4	34.16
312	1	AFC	4	32.97
313	1	AFC	4	35.04
318	1	AFC	4	27.41
326	1	NSP-0.125%	4	28.63
330	1	NSPC	4	33.65
334	1	NSP-0.25%	4	30.50
336	1	NSPC	4	35.19
340	1	NSPC	4	21.91
845	1	NSP-0.25%	4	31.02
862	1	NSP-0.25%	4	37.91
864	1	NSP-0.125%	4	36.85
895	1	CON	4	35.38
941	1	NSP-0.125%	4	32.16
306	1	CON	5	28.33
309	1	CON	5	33.63
312	1	AFC	5	34.89
313	1	AFC	5	30.95
318	1	AFC	5	23.53
326	1	NSP-0.125%	5	25.94
330	1	NSPC	5	33.78
334	1	NSP-0.25%	5	30.45
336	1	NSPC	5	30.83
340	1	NSPC	5	28.22
845	1	NSP-0.25%	5	34.40
862	1	NSP-0.25%	5	36.23
864	1	NSP-0.125%	5	41.39
895	1	CON	5	34.06
941	1	NSP-0.125%	5	30.80

Table B.1 (Continued)

306	2	NSP-0.125%	11	29.48
309	2	NSP-0.125%	11	32.12
312	2	CON	11	36.14
313	2	CON	11	35.69
318	2	CON	11	24.41
326	2	NSP-0.25%	11	28.61
330	2	AFC	11	33.79
334	2	NSPC	11	26.50
336	2	AFC	11	30.71
340	2	AFC	11	28.17
845	2	NSPC	11	28.89
862	2	NSPC	11	22.69
864	2	NSP-0.25%	11	37.10
895	2	NSP-0.125%	11	32.70
941	2	NSP-0.25%	11	31.69
306	2	NSP-0.125%	12	30.30
309	2	NSP-0.125%	12	29.52
312	2	CON	12	35.56
313	2	CON	12	33.74
318	2	CON	12	23.81
326	2	NSP-0.25%	12	25.52
330	2	AFC	12	30.35
334	2	NSPC	12	25.59
336	2	AFC	12	28.68
340	2	AFC	12	28.66
845	2	NSPC	12	32.83
862	2	NSPC	12	38.08
864	2	NSP-0.25%	12	32.45
895	2	NSP-0.125%	12	30.06
941	2	NSP-0.25%	12	30.52
306	2	NSP-0.125%	13	31.12
309	2	NSP-0.125%	13	33.20
312	2	CON	13	38.10
313	2	CON	13	36.09
318	2	CON	13	25.28
326	2	NSP-0.25%	13	24.17
330	2	AFC	13	33.85
334	2	NSPC	13	30.14
336	2	AFC	13	31.37

Table B.1 (Continued)

340	2	AFC	13	27.53
845	2	NSPC	13	30.40
862	2	NSPC	13	35.21
864	2	NSP-0.25%	13	34.68
895	2	NSP-0.125%	13	28.46
941	2	NSP-0.25%	13	32.44
306	2	NSP-0.125%	14	28.82
309	2	NSP-0.125%	14	31.01
312	2	CON	14	40.55
313	2	CON	14	35.75
318	2	CON	14	25.27
326	2	NSP-0.25%	14	23.10
330	2	AFC	14	33.01
334	2	NSPC	14	22.80
336	2	AFC	14	34.09
340	2	AFC	14	28.74
845	2	NSPC	14	31.65
862	2	NSPC	14	35.32
864	2	NSP-0.25%	14	31.31
895	2	NSP-0.125%	14	28.44
941	2	NSP-0.25%	14	28.57
306	2	NSP-0.125%	15	29.30
309	2	NSP-0.125%	15	31.55
312	2	CON	15	42.37
313	2	CON	15	37.62
318	2	CON	15	20.11
326	2	NSP-0.25%	15	22.97
330	2	AFC	15	34.01
334	2	NSPC	15	21.45
336	2	AFC	15	34.47
340	2	AFC	15	27.34
845	2	NSPC	15	29.63
862	2	NSPC	15	37.83
864	2	NSP-0.25%	15	31.22
895	2	NSP-0.125%	15	25.61
941	2	NSP-0.25%	15	30.30
306	3	NSP-0.25%	21	35.02
309	3	NSP-0.25%	21	44.33
312	3	NSP-0.125%	21	46.27

Table B.1 (Continued)

313	3	NSP-0.125%	21	50.58
318	3	NSP-0.125%	21	32.54
326	3	NSPC	21	29.55
330	3	CON	21	45.45
334	3	AFC	21	32.96
336	3	CON	21	38.16
340	3	CON	21	34.40
845	3	AFC	21	41.88
862	3	AFC	21	44.26
864	3	NSPC	21	41.84
895	3	NSP-0.25%	21	41.93
941	3	NSPC	21	40.73
306	3	NSP-0.25%	22	31.09
309	3	NSP-0.25%	22	44.16
312	3	NSP-0.125%	22	47.73
313	3	NSP-0.125%	22	49.97
318	3	NSP-0.125%	22	28.69
326	3	NSPC	22	30.06
330	3	CON	22	42.19
334	3	AFC	22	30.48
336	3	CON	22	36.82
340	3	CON	22	33.87
845	3	AFC	22	39.48
862	3	AFC	22	48.75
864	3	NSPC	22	43.37
895	3	NSP-0.25%	22	37.83
941	3	NSPC	22	39.04
306	3	NSP-0.25%	23	32.57
309	3	NSP-0.25%	23	46.44
312	3	NSP-0.125%	23	47.67
313	3	NSP-0.125%	23	53.35
318	3	NSP-0.125%	23	27.93
326	3	NSPC	23	28.28
330	3	CON	23	41.06
334	3	AFC	23	28.08
336	3	CON	23	39.05
340	3	CON	23	32.37
845	3	AFC	23	37.78
862	3	AFC	23	45.32

Table B.1 (Continued)

864	3	NSPC	23	39.26
895	3	NSP-0.25%	23	39.52
941	3	NSPC	23	40.91
306	3	NSP-0.25%	24	29.64
309	3	NSP-0.25%	24	50.37
312	3	NSP-0.125%	24	44.53
313	3	NSP-0.125%	24	50.50
318	3	NSP-0.125%	24	27.74
326	3	NSPC	24	28.60
330	3	CON	24	38.26
334	3	AFC	24	29.14
336	3	CON	24	39.12
340	3	CON	24	28.90
845	3	AFC	24	37.30
862	3	AFC	24	44.30
864	3	NSPC	24	40.01
895	3	NSP-0.25%	24	36.11
941	3	NSPC	24	38.91
306	3	NSP-0.25%	25	31.99
309	3	NSP-0.25%	25	44.81
312	3	NSP-0.125%	25	46.27
313	3	NSP-0.125%	25	49.02
318	3	NSP-0.125%	25	25.23
326	3	NSPC	25	24.82
330	3	CON	25	38.56
334	3	AFC	25	28.50
336	3	CON	25	34.58
340	3	CON	25	27.88
845	3	AFC	25	33.71
862	3	AFC	25	42.80
864	3	NSPC	25	35.57
895	3	NSP-0.25%	25	38.08
941	3	NSPC	25	36.85
306	4	NSPC	31	25.00
309	4	NSPC	31	46.47
312	4	NSP-0.25%	31	48.75
313	4	NSP-0.25%	31	47.72
318	4	NSP-0.25%	31	29.52
326	4	AFC	31	26.84

Table B.1 (Continued)

330	4	NSP-0.125%	31	39.38
334	4	CON	31	17.16
336	4	NSP-0.125%	31	32.25
340	4	NSP-0.125%	31	23.72
845	4	CON	31	34.90
862	4	CON	31	39.59
864	4	AFC	31	34.85
895	4	NSPC	31	32.91
941	4	AFC	31	34.96
306	4	NSPC	32	27.50
309	4	NSPC	32	46.06
312	4	NSP-0.25%	32	44.80
313	4	NSP-0.25%	32	47.15
318	4	NSP-0.25%	32	28.74
326	4	AFC	32	26.14
330	4	NSP-0.125%	32	38.47
334	4	CON	32	19.70
336	4	NSP-0.125%	32	31.33
340	4	NSP-0.125%	32	24.36
845	4	CON	32	34.04
862	4	CON	32	39.68
864	4	AFC	32	33.49
895	4	NSPC	32	32.23
941	4	AFC	32	36.01
306	4	NSPC	33	29.33
309	4	NSPC	33	42.94
312	4	NSP-0.25%	33	45.57
313	4	NSP-0.25%	33	44.40
318	4	NSP-0.25%	33	28.72
326	4	AFC	33	25.13
330	4	NSP-0.125%	33	40.33
334	4	CON	33	18.22
336	4	NSP-0.125%	33	31.66
340	4	NSP-0.125%	33	23.84
845	4	CON	33	34.80
862	4	CON	33	39.60
864	4	AFC	33	35.25
895	4	NSPC	33	35.82
941	4	AFC	33	36.51

Table B.1 (Continued)

306	4	NSPC	34	25.76
309	4	NSPC	34	46.54
312	4	NSP-0.25%	34	45.52
313	4	NSP-0.25%	34	43.43
318	4	NSP-0.25%	34	26.92
326	4	AFC	34	22.74
330	4	NSP-0.125%	34	37.07
334	4	CON	34	21.44
336	4	NSP-0.125%	34	32.64
340	4	NSP-0.125%	34	24.01
845	4	CON	34	34.44
862	4	CON	34	39.08
864	4	AFC	34	34.93
895	4	NSPC	34	35.10
941	4	AFC	34	34.11
306	4	NSPC	**35**	.
309	4	NSPC	**35**	.
312	4	NSP-0.25%	**35**	.
313	4	NSP-0.25%	**35**	.
318	4	NSP-0.25%	**35**	.
326	4	AFC	**35**	.
330	4	NSP-0.125%	**35**	.
334	4	CON	**35**	.
336	4	NSP-0.125%	**35**	.
340	4	NSP-0.125%	**35**	.
845	4	CON	**35**	.
862	4	CON	**35**	.
864	4	AFC	**35**	.
895	4	NSPC	**35**	.
941	4	AFC	**35**	.
306	5	AFC	41	27.12
309	5	AFC	41	45.45
312	5	NSPC	41	45.59
313	5	NSPC	41	47.28
318	5	NSP	41	23.62
326	5	CON	41	24.68
330	5	NSP-0.25%	41	42.83
334	5	NSP-0.125%	41	18.61
336	5	NSP-0.25%	41	37.52

Table B.1 (Continued)

340	5	NSP-0.25%	41	25.44
845	5	NSP-0.125%	41	34.13
862	5	NSP-0.125%	41	37.99
864	5	CON	41	34.82
895	5	AFC	41	33.00
941	5	CON	41	35.51
306	5	AFC	42	30.09
309	5	AFC	42	42.47
312	5	NSPC	42	43.88
313	5	NSPC	42	44.91
318	5	NSPC	42	23.84
326	5	CON	42	25.39
330	5	NSP-0.25%	42	35.57
334	5	NSP-0.125%	42	19.03
336	5	NSP-0.25%	42	32.98
340	5	NSP-0.25%	42	26.06
845	5	NSP-0.125%	42	34.43
862	5	NSP-0.125%	42	36.89
864	5	CON	42	33.81
895	5	AFC	42	33.95
941	5	CON	42	33.48
306	5	AFC	43	31.02
309	5	AFC	43	43.22
312	5	NSPC	43	44.84
313	5	NSPC	43	47.25
318	5	NSPC	43	24.48
326	5	CON	43	24.81
330	5	NSP-0.25%	43	37.80
334	5	NSP-0.125%	43	19.91
336	5	NSP-0.25%	43	34.79
340	5	NSP-0.25%	43	25.75
845	5	NSP-0.125%	43	33.28
862	5	NSP-0.125%	43	37.94
864	5	CON	43	35.22
895	5	AFC	43	35.37
941	5	CON	43	34.54
306	5	AFC	44	29.00
309	5	AFC	44	43.87
312	5	NSPC	44	43.32

Table B.1 (Continued)

313	5	NSPC	44	47.09
318	5	NSPC	44	23.53
326	5	CON	44	24.80
330	5	NSP-0.25%	44	37.79
334	5	NSP-0.125%	44	12.98
336	5	NSP-0.25%	44	34.12
340	5	NSP-0.25%	44	24.75
845	5	NSP-0.125%	44	32.36
862	5	NSP-0.125%	44	39.64
864	5	CON	44	37.32
895	5	AFC	44	34.61
941	5	CON	44	33.01
306	5	AFC	45	28.04
309	5	AFC	45	46.37
312	5	NSPC	45	39.08
313	5	NSPC	45	42.81
318	5	NSPC	45	21.63
326	5	CON	45	22.06
330	5	NSP-0.25%	45	36.32
334	5	NSP-0.125%	45	18.05
336	5	NSP-0.25%	45	12.97
340	5	NSP-0.25%	45	23.11
845	5	NSP-0.125%	45	31.67
862	5	NSP-0.125%	45	38.52
864	5	CON	45	34.24
895	5	AFC	45	32.47
941	5	CON	45	31.78

[1]NSP (BASF Corp., Ludwigshaven, Germany)
[2]CON = 0 ppb AFB_1 + 0 g NSP, AFC = 50 ppb AFB_1 + 0% DM NSP, NSPC = 0 ppb AFB_1 + 0.5% DM NSP, NSP-0.125% = 50 ppb AFB_1 + 0.125% DM NSP, NSP-0.25% = 50 ppb AFB_1 + 0.25% DM NSP