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Assessment of aflatoxin B_1 adsorption efficacy of natural and processed bentonites: In vitro and in vivo assays

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ABSTRACT

The presence of aflatoxin B_1 in livestock's feed results in contamination of milk and its products with aflatoxin M_1. Different sequestering agents have been added to cows' ration to adsorb these toxins, although influence of processed bentonites on aflatoxins adsorption has not been evaluated yet. This experiment was carried out to assess the effects of incorporating natural and processed bentonite (local or commercially available), to the diet of Holstein dairy cows subjected to an aflatoxin B_1 diet, and the transfer of aflatoxin metabolites (AFM_1) to milk. Aflatoxin sequestering capacity, pH, CEC, XRD and XRF of natural and processed bentonites were measured. Then, twelve Holstein dairy cows were assigned to 3 treatments as the following: 1) local processed bentonite (G.Bind™), 2) local unprocessed bentonite (F), and 3) commercially available bentonite (M). Aflatoxin content in feed and milk was evaluated and transfer rate was measured. Results of the present study showed that the aflatoxin contents of milk were remained unchanged except for treatment G.Bind™ that considerably decreased aflatoxin M_1 in milk after the second and third weeks of the experiment. G.Bind™ lowered the transfer rate of aflatoxin B_1 from 1.17% at the beginning of the experiment to 0.43% and 0.39% after the first and second weeks, respectively. Processing of bentonites (basic processing in present study) can considerably help to adsorb aflatoxin from feed and also to decrease aflatoxin transfer to milk.

1. Introduction

A group of fungi can produce toxic secondary metabolites known as mycotoxins, which have detrimental effects on human health and animal performance (Bryden, 2012; Jard et al., 2011; Robens and Richard, 1992). The Food and Agriculture Organization (FAO) reported that about 25% of crops produced all over the world are contaminated with mycotoxins (Fokunang et al., 2006). Aflatoxins are the most common and harmful metabolites, which are produced by different strains of *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus* (Heathcote and Hibbert, 1978). Aflatoxins detrimentally affect hepatocytes (Schlemper et al., 1991), decrease milk production (Malka et al., 2013), decline egg production (Hamilton and Garlich, 1971), attenuate immune response and resistance against pathogens (Ghosh et al., 1990; Bondy and Pestka, 2000), and dramatically depress performance (Kermanshahi et al., 2009; Dersjant-Li et al., 2003) in farm animals. Among different members of the aflatoxin family, aflatoxin B_1 (AFB_1) possesses the most toxic and carcinogenic characteristics to animals and humans (McLean and Dutton, 1995). About 5 billion humans in different countries are at risk of AFB_1 through different contaminated food and livestock products (Liu and Wu, 2010).

AFB_1 can be partially destroyed in the rumen if ruminants are fed with the contaminated feedstuffs, although AFB_1 may absorb and undergo different metabolic processes to transform to other metabolites in the liver (Kuilman et al., 1998). The most common metabolite of AFB_1 is aflatoxin M1 (AFM_1) which can be excreted in the urine and milk in different amounts based on the AFB_1 existing in feedstuffs (Prandini et al., 2007). AFM_1 is stable and remains unaffected by common milk processing procedures, such as pasteurization and sterilization. This metabolite can be transported to different dairy products and increase the aflatoxin exposure. The International Agency for Research on Cancer (IARC, 2002) classified AFB_1 as a type I carcinogenic agent and AFM_1 as type II. Health organizations in different parts of world have established minimum levels of AFM_1 in dairy products; for example, the European Union (EU, 2006) applies 0.05 μg AFM_1 per kg in ruminant milk as the maximum residual level, while in the USA, some Asian and South American counties accept a higher level (0.5 μg AFM_1/kg ruminant milk) (Binder et al., 2007).

About 1% to 3% of AFB_1 existing in feedstuffs appear in milk as AFM_1 (Diaz et al., 2004). So, different methods have been applied to sequester AFB_1 in feed and subsequently prevent transformation to AFM_1 and presence in milk. Some of these methods comprise irradiation,

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biological detoxification, chemical detoxification, and deactivation by heat. Although these methods are successful in decreasing AFB_1 in feedstuffs, they might also depress the nutritional value of dairy products (Phillips et al., 1994). Clay based adsorbents are the most efficient ones in adsorbing AFB_1 (Phillips, 1999). Among different clay adsorbents, special attention is paid to bentonite and its main mineral, montmorillonite (Mt). The layer structure of Mt allows swelling in aqueous environment, promoting an AFB_1 adsorption between the layers and prevents the absorbance of these toxic molecules by gut cells (Diaz et al., 2004: Eckhardt et al., 2014). Deng et al. (2010) reviewed different possible mechanisms by which clays may adsorb AFB_1 molecules; these mechanisms mainly contain chemical bonds between active sites in AFB_1 and clays. Based on the in vitro results (Diaz et al., 2002), which showed the beneficial effects of clays on sequestering AFB_1, animal nutritionists have tended to incorporate clays to livestock's diet contaminated with AFB_1 to examine the effects of such additives on AFB_1 decontamination and animals' performance. Mt successfully adsorbed AFB_1 in the gastrointestinal tract (Eckhardt et al., 2014), reduced hepatic lesions and mortality (Pasha et al., 2007), reduced AFM_1 in milk (Diaz et al., 2004), and improved performance (Shi et al., 2006). It is also well-documented that natural Mt, without processing markedly adsorbed AFB_1 under in vitro and in vivo conditions (Dakovic et al., 2008; Phillips et al., 1988). Although several studies were conducted to assess the sequestering effects of natural Mt on AFB_1, there are few reported studies that compare natural vs. activated Mt on AFB_1 decontamination. The objective of present study was to evaluate the effects of activated clay in comparison to both non-activated and commercially available clay binders on AFB_1 adsorption in dairy cows' ration and transport rate from feed to milk.

2. Materials and methods

2.1. Animals, treatments, experimental conditions and sampling

Twelve Holstein dairy cows with similar physiological and productive attributes (average body weight of 480 ± 55 kg; average age of 60 ± 8 months; average milk production of 45 ± 6 kg) were used for two weeks in this experiment. All dairy cows were assigned to three groups and fed the same total mixed ration (TMR) for a week before the beginning of the experiment in order to bear the adaptation period. The experimental diets were: 1) unprocessed Mt (F), 2) imported commercially available clay (M), and 3) local processed Mt (G.Bind™,[1]). A source of cottonseed meal, which had been naturally contaminated with AFB_1, was added to the diets, completely mixed and samples were collected and tested to confirm that all cows received the same dose of AFB_1 during the two weeks of the experiment. Then, all experimental clay binders were added to the diets according to recommendations of producing companies (i.e. 0.6%), at the expense of corn and thoroughly mixed. The diet was formulated according to the recommendation of NRC, 2001 (Table 1) for an average cow weighing 450 kg, 160 days in lactation and with a 40 kg milk yield (3.8% fat, 3.35% protein). The TMR was fed ad libitum twice a day at 10:00 h and 16:00 h with checking that no empty trough was observed. Cows were milked 3 times a day at 3:30, 11:30 and 19:30. The experimental protocols were reviewed and approved by the Animal Care Committee of the Ferdowsi University of Mashhad, Iran.

2.2. Bentonite characteristics

Clay samples were characterized by X-ray Fluorescence (XRF) using an XMF104 Unisantis Electronics spectrophotometer operating at a power of 1 kW and equipped with an Rh X-ray source. The clay samples were prepared as random powders to record the X-ray diffraction patterns on a XMD300, Unisantis Electronics diffractometer employing Cu Kα radiation (λ = 1.54 Å) with a rotating sample stage and a fixed divergence slit size of 0.5°. The samples were scanned between 3 and 65° 2θ. The step size was 0.02° 2θ and counting time was 80 s per step. Physical attributes of bentonites such as cation exchange capacity (CEC: according to ASTM[2] C837), swelling (according to ASTM D5890), water absorption (according to ASTM E946-92), methylene blue index (according to ASTM C837-09), pH (according to ASTM D4972-13) and percentage of <2 μ particles with particle size analyzer (Sedigraph 5100, Micromeritics, GA, USA) were analyzed.

Table 1
Ingredient and nutrient composition of the TMR fed to lactating dairy cows based on NRC (2001)[a].

Ingredients	(%)
Alfalfa Hay	10.0
Corn silage	59.0
Ground corn	6.0
Barley	8.0
Cottonseed	2.0
Soybean meal	6.5
Cottonseed meal	1.0
Sunflower meal	1.5
Meat meal	3.0
Fat meal	1.5
Sodium bicarbonate	0.2
DCP	0.6
Mineral and vitamin supplements[b]	0.5
Salt	0.2
Nutrients	
NE_L (Mcal/kg)	1.63
CP (%)	18.40
RUP (%CP)	6.60
RDP (%CP)	11.80
NFC (%)	38.10
ADF (%)	20.00
NDF (%)	21.00
Forage NDF (%)	21.30
Ca (%)	1.00
P (%)	0.70
DCAD (meq/kg)	188.00

[a] Clay samples were added to the diets in the expense of corn according to the recommendation of producing factory.
[b] Each kilogram of mineral and vitamin supplement contains: 190,000 mg Ca, 90,000 mg P, 50,000 mg Na, 19,000 mg Mg, 3000 mg Fe, 300 mg Cu, 3000 mg Zn, 100 mg Co, 100 mg I, 1 mg Se, 3000 antioxidant, 50,000 IU vit. A, 100,000 IU vit. D_3, and 1000 mg vit. E.

2.3. Sample preparation and AFB_1 and AFM_1 measurement

At the beginning of the experiment, a sample of the TMR was collected, oven dried at 60 °C until constant weight, ground and frozen until AFB_1 analysis. Three samples of milk obtained from each milking time for each group were collected in sterile 15 ml propylene falcon at the first day of experiment and also at the end of each week (two weeks). Collected samples were stored at 4 °C and frozen at −20 °C for later AFM_1 analysis.

For measuring AFB_1 in the feed samples, 6 replicates of 5 g (total of 30 g) of the TMR sample were thawed, passed through 1 mm sieve, oven-dried and sonicated in 25 ml methanol: water (70:30 v/v) solution in an ultrasonic bath for 10 min. The obtained solution was passed through a Whatman No. 1 filter paper, transferred to enzyme-linked immunosorbent assay (ELISA) kit, following the instructions of the kit producer (Neogen, USA) and finally the prepared kit was placed in ELISA (BioTek, ELX 808, Winooski, VT, USA). Ten wells were assigned for each replicate of the TMR sample (total of 60 wells) and average data

[1] G.Bind™ is treated with a basic solution to activate clay through sodium entrance to the interlayer of montmorillonite to improve physical characteristics of clay. G.Bind™ is a product of PayaFarayand Hezareh Novin micronized mineral manufacturing, Mashhad, Razavi Khorasan, Iran.

[2] American Society for Testing and Materials, West Conshohocken, Pennsylvania, United States.

Table 2
Mineralogical analysis of different experimental bentonites examined by X-ray diffraction (XRD).

Clay samples	F[a]	G.Bind™	M
Minerals	Montmorillonite, cristobalite, quartz, gypsum, calcite	Montmorillonite, cristobalite	Montmorillonite, quartz

[a] F: unprocessed clay; G.Bind™: basic processed clay; M: commercially available clay.

were used. The concentration of AFB_1 detected in the TMR sample of first day of experiment (1.95 ppb) was defined as base level of AFB_1 for all experimental groups.

For measuring AFM_1, milk samples were thawed and centrifuged at 2000 × *g* for 10 min at 25 °C. After centrifugation, the fat layer was removed and remained milk was used to quantify aflatoxin with an ELISA test kit (EuroProxima AFM_1, Arnhem, Netherland). Ten wells were assigned for each milk sample and average data were used. The level of AFM_1 detected in the milk samples collected at the first day of experiment (153, 159 and 162 ppt for G.Bind, F and M, respectively) was defined as base level of AFM_1 for all experimental groups.

2.4. Calculation of AFM_1 transfer rate

To determine AFM_1 transfer rate to milk, data was collected and measured according to Roxane (2010). Briefly, AFM_1 excretion was calculated as the following formula:[3]

$$\text{AFM}_1 \text{ excretion} = (\text{concentration of AFM}_1 \text{ in milk}) \times (\text{average amount of milk produced}).$$

Then, the aflatoxin transfer rate was measured through the formula below:

$$\text{AFM}_1 \text{ transfer rate} = \frac{\text{AFM}_1 \text{ excretion}}{\text{AFB}_1 \text{ consumption}}.$$

2.5. Statistical analysis

Data were analyzed by the General Linear Models procedure of SAS (SAS Institute, 2004) as a completely randomized experimental design. The analysis of variance was compared using Tukey's Honestly Significant Difference (HSD). Difference between means was significant when $P < 0.05$.

3. Results

The mineral identification from the XRD patterns is shown in Table 2. Montmorillonite was the dominant mineral in all samples which could affect toxin adsorption capacity and other physicochemical characteristics.

Chemical analysis of clay samples is summarized in Table 3. The most abundant elements present in clay samples were Si, O and Al, while Fe, K, Mg and Mn were only found in small quantities (less than 15%).

Physicochemical analysis of clay samples is presented Table 4. The basic processing performed on G.Bind™ with sodium ions added to Mt interlayer space led to two important changes on G.Bind™ that are in favor of absorbing toxin molecules; firstly, pH values in G.Bind™ (9.8) increased compared to F (8.7) and M (7.8) groups. Secondly, entering sodium ions into the interlayer space resulted in increasing water absorption capacity and swelling index of G.Bind™ (615% and 19 ml/2 g, respectively) in comparison to F (335% and 10 ml/2 g, respectively) and M (265% and 5 ml/2 g, respectively).

The effects of the three different bentonites on milk AFM_1 concentration in consecutive weeks are shown in Table 5. There was no significant difference between milk AFM_1 concentrations of cows designated to different groups at the beginning of the experiment. Although AFM_1 declined in all experimental groups, G.Bind™ bentonite decreased AFM_1 significantly ($P < 0.05$) as compared to M and F at the end of the first week. Similar results were observed for the second week when compared to the first week; G.Bind™ lowered significantly ($P < 0.05$) AFM_1 when compared to the M and F groups.

The transfer rate of AFM_1 in different experimental groups in the period of two weeks is summarized in Table 6. Although all treatments had relatively similar transfer rates at the beginning of the experiment, cows that received G.Bind™ showed lower ($P < 0.05$) AFM_1 transfer rate to milk as compared to the F and M groups.

4. Discussion

Aflatoxins are categorized as carcinogenic secondary metabolites of fungi *Aspergillus spp.* (IARC, 2002); so, the presence of aflatoxins in food products can threaten human health. One of the commonest sources of food for human is dairy products, which are directly or indirectly provided from farm animals' milk. Since aflatoxin-contaminated feedstuffs can detrimentally affect host animals, toxins can transfer to milk and subsequently dairy products and negatively affect human health. Therefore, many adsorbents have been proposed to sequester toxin molecules in animal body. Among, clay minerals, especially Mt, has attracted much scientific interest due to the physico-chemical properties. Masimango et al. (1978) firstly used Mt to sequester aflatoxin and alleviate toxicity in vitro. Since then, many studies have been conducted to find the mechanisms by which clay minerals, especially Mt, can absorb aflatoxin molecules in their structures. Phillips (1999) proposed that aflatoxin molecules can absorb onto Mt on three different types of sites which comprise the external basal surfaces, edges, and the interlayer space, where the last position seems the most important site to absorb aflatoxin molecules. Two carbonyl groups in the aflatoxin molecule are the active sites with partial positive charges that define the amount of affinity of toxin molecule to sorbents (Theng, 1974). Different mechanisms by which aflatoxin molecules can absorb to Mt have been proposed, which include electron donor–acceptor model, selective chemisorption, and hydrogen bonding. In electron donor–acceptor model, the partial positive charges on aflatoxin molecules might share electrons with the negative charges on the Mt surface which, leads to absorbance of toxin to Mt (Phillips, 1999). In the selective chemisorption concept, the enthalpy of reaction between aflatoxin molecule and Mt shows that active carbonyl sites in aflatoxin can form a chelate with transition metals in Mt and subsequently sequester toxin molecules (Phillips et al., 1995). The last possible mechanism is the formation of a hydrogen bond between the toxin molecule and Mt exchange cations in the interlayer space (Tenorio et al., 2008). Deng et al. (2010) reported that the electron donor–acceptor model is the dominant mechanism in dry conditions, while the formation hydrogen bond between active sites on the toxin molecule and Mt is the most important model in wet conditions to describe the sequestering effect of clay minerals. Deng and Szczerba (2011) demonstrated that the computational simulation of bonding between Mt and aflatoxin confirmed the importance of linkage between active site of aflatoxin molecule (i.e. carbonyl groups) and active sites of Mt (i.e. exchangeable cations in interlayer space) to absorb toxin on Mt. In addition, Deng et al. (2012) reported that the capacity of Mt for absorbing aflatoxin molecules can change through replacing exchangeable cations. So, there is a continuous effort to process clay minerals, especially Mt, to activate and improve cations

[3] Milk production data are presented in supplemental file.

Table 3
Chemical analysis (%) of different experimental clay samples examined by X-ray fluorescence (XRF).

	F[a]	G.Bind™	M
SiO_2	60.11	65.14	61.16
Al_2O_3	10.10	10.34	13.99
Fe_2O_3 (T)	2.89	2.26	3.87
MgO	3.28	2.10	2.06
CaO	3.12	2.66	1.62
P_2O_5	0.09	0.06	0.03
Na_2O	2.34	2.67	0.32
K_2O	0.39	0.30	0.57
MnO	0.61	0.41	0.08
SO_3	2.76	0.72	0.62
L.O.I[b]	13.53	12.34	14.90

[a] F: unprocessed bentonite; G.Bind™: basic processed bentonite; M: commercially available imported bentonite.
[b] Loss of ignition.

in the interlayer space to increase the adsorption capacity. Dwyer et al. (1997) used hydrochloric acid to produce acidic Mt and then added it to broiler diets, but no significant effects on mycotoxin adsorption were observed by these researchers. In contrast, Diaz et al. (2004) and Stroud (2006) demonstrated that bentonites can adsorb aflatoxin molecules in their structure in mild basic pH such as 8 and 9. In the present study, a basic processed clay (G.Bind™) was utilized to compare its AFB_1 adsorption capacity to unprocessed (F) and commercially available products (M) in cows fed with AFB_1 contamination feed. Although clay samples used in the present study had diluting components such as quartz, calcite and gypsum (Table 2), which might interfere in absorbing toxin molecules, Magnoli et al. (2008) reported that these components had no effect on aflatoxin adsorption. In the process of producing G.Bind™, a slurry of sodium carbonate was added to clay which leads to establishment of sodium ions in the interlayer space and therefore the charge density increased in this space, which according to Deng et al. (2012), increased the selectivity of Mt for aflatoxin molecules by enhancing charge density.

As described before, the basic processing in G.Bind™ caused both an increase in pH value, swelling index and water absorption capacity (Table 4) compared to F and M groups. Increase in pH value led to beneficially preparing G.Bind™ structure for accepting aflatoxin molecules (Deng et al., 2012). In addition to increasing pH, the basic processing resulted in enlargement of the interlayer space in Mt that helped to easily absorb more aflatoxins in this space (Phillips, 1999). In contrast to G.Bind™, F group had no effects on aflatoxin adsorption. In fact, F is the unprocessed form of G.Bind™ directly obtained from mines and then underwent basic processing to produce G.Bind™. Comparing results of G.Bind™ and F groups confirmed that basic processing is a reliable method to improve the quality of a clay to absorb AFB_1 in animal feed. The M clay producer claimed that this clay was specially processed in order to absorb toxins from animal feedstuffs. Roxane (2010) evaluated the effects of some commercially available bentonites on AFM_1 adsorption in milking cows and pigs. In agreement with the present results, this researcher reported that processed calcium bentonite had higher adsorption capacity in comparison to other unprocessed clays.

Table 5
Effect of experimental bentonites on aflatoxin M_1 concentration (ppt) in lactating cows' milk.

Clay samples[1]	Beginning of experiment	End of first week	End of second week
G.Bind™	153[a]	54[b]	47[b]
F	159[a]	140[a]	149[a]
M	162[a]	156[a]	156[a]
SEM[2]	10.6	34.3	36.8

[a–b]Means sharing a common superscript in a column do not differ significantly ($P > 0.05$).
[1] G.Bind™: basic processed bentonite; F: unprocessed bentonite; M: commercially available imported bentonite.
[2] SEM: standard errors of means.

Table 6
Effect of experimental bentonites on transfer rate (%) of aflatoxin M_1 to lactating cows' milk.

Clay samples[1]	Beginning of experiment	End of first week	End of second week
G.Bind™	1.17	0.42[b]	0.39[b]
F	1.30	1.15[a]	1.24[a]
M	1.18	1.10[a]	1.18[a]
SEM[2]	1.14	0.26	0.32

[a–b]Means sharing a common superscript in a column do not differ significantly ($P > 0.05$).
[1] G.Bind™: basic processed bentonite; F: unprocessed bentonite; M: commercially available imported bentonite.
[2] SEM: Standard errors of means.

Stroud (2006) investigated the effects of one commercial processed bentonite (Novasil) on AFM_1 transfer rate and revealed that this adsorbent could beneficially decrease aflatoxin concentrations in cow's milk, which is in consistent with the present results. In fact, adsorption of aflatoxin molecules onto Mt in the aqueous environment of rumen in cow resulted in lowering toxin adsorption by animal intestine, decreasing aflatoxins received by the liver and finally secreting less AFM_1 in milk (Diaz et al., 2004).

5. Conclusion

Overall, basic processing of clays may increase exchanged cations in the interlayer spaces in Mt and improve the ability of clays to sequester aflatoxin molecules which prevents the adsorption of toxins from animal intestine and subsequently decrease transfer rate of AFB_1 from the liver to milk in lactating cows which enhances milk quality and leads to healthier dairy products for consumers. Although G.Bind™ (the product of basic processing) could dramatically decrease the concentration of AFM_1 in milk to the lesser amount, more investigations should be conducted to examine different activation methods to increase the potency of clays to sequester various mycotoxins.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clay.2016.01.019.

Table 4
Physicochemical analysis of different experimental bentonites examined according to American Society for Testing and Materials (ASTM) methods.

Clay samples[a]	pH[b]	CEC (meq/100 g)	<2 μ particles (%)	Water absorption (%) (2 h)	Swelling (ml/2 g)	Methylene blue index (%)
G.Bind™	9.8	130.8	70.4	615.0	19.0	75.0
F	8.7	140.0	72.0	335.0	10.0	70.0
M	7.8	71.7	53.0	265.0	5.0	57.2

[a] G.Bind™: basic processed bentonite; F: unprocessed bentonite; M: commercially available imported bentonite.
[b] pH (according to ASTM D4972-13), cation exchange capacity (CEC: according to ASTM C837), swelling (according to ASTM D5890), water absorption (according to ASTM E946-92), methylene blue index (according to ASTM C837-09).

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