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# Effect of Feeding Bentonite Clay on Excretion of Aflatoxin $M_1$ in Milk of Dairy Animals

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## ABSTRACT

Aim of this field study was to evaluate the effect of feeding sodium bentonite on feed intake, milk yield, excretion of aflatoxin $M_1$ and mineral bioavailability in dairy animals. Thirty multiparous lactating cross-bred cows (n=26) and buffaloes (n=4) having milk yield 10-15 kg/day were divided into three groups (Control, T1 and T2) of ten each, based on milk yield, fat% and stage of lactation (90-180 post calving). All animals were daily fed basal diet comprising 3-4 kg cattle feed, 2-3 kg maize bhardo (ground maize grain), 15-20 kg hybrid napier fodder and 4-5 kg jowar straw. In addition to basal diet, each animal in treatment groups T1 and T2 were daily supplemented 100 and 150 g sodium bentonite clay (montmorillonite-87.67%;*in vitro* net binding capacity-97.6%), respectively. Dry matter intake, milk yield, aflatoxin $B_1$ intake and excretion of aflatoxin $M_1$ of individual animals were recorded on day 0, 15, 30, 45 and 60. Blood samples were collected before (0 day) and after (60 days) feeding sodium bentonite. Study revealed that supplementation of sodium bentonite did not affect ($P>0.05$) dry matter intake and milk yield. Average aflatoxin $B_1$ intake was 83.1, 89.4 and 105.9 µg/kg dry matter intake in control, T1 and T2 groups, respectively. Supplementation of sodium bentonite @ 100 and 150 g in the ration of dairy animals significantly ($P<0.05$) reduced aflatoxin $M_1$ in milk by 61.6% and 54.2%, respectively. Transfer rate of aflatoxin from feed to milk also reduced significantly($P<0.05$) by 48.2% and 43.9% in T1 and T2 groups, respectively. Blood mineral profiles did not affect ($P>0.05$) due to supplementation of bentonite in dairy animals. Daily supplemental cost of commercial bentonite clay was less than Rs. 1.0 per animal. Study demonstrates that supplementation of bentonite clay help to reduce aflatoxin $M_1$ in milk, without affecting feed intake, milk yield and blood mineral profiles in dairy animals.

**KEY WORDS:** Aflatoxins, Bentonite, Milk, Mineral bioavailability, Ruminants

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## INTRODUCTION

Aflatoxins are naturally occurring secondary metabolites produced mainly by toxicogenic strains of *Aspergillus flavus* and *A. parasiticus* that colonize crops, including many dietary staple foods (maize, corn, groundnuts, and rice) and feed ingredients. These fungi usually grow in warm and humid climatic conditions of tropical and subtropical regions, such as India (Battilani et al., 2011). Aflatoxins occur in feeds as aflatoxin $B_1$ ($AFB_1$), $B_2$, $G_1$ and $G_2$ forms and in milk as their oxidative forms: $M_1$ and $M_2$. Both aflatoxin $B_1$ ($AFB_1$) and aflatoxin $M_1$ ($AFM_1$) are carcinogenic; however, $AFM_1$ is the most toxic secondary metabolite that is secreted into milk and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2002). $AFM_1$ represents a food safety risk to humans (Gallo et al., 2015) and food processing techniques are not sufficient to eliminate aflatoxins from contaminated feed due to their heat resistant nature (Medina et al., 2017).

Several approaches have been investigated to reduce exposure of animals to aflatoxins in contaminated feeds. The most common and practical approach for control of $AFM_1$ in milk is use of appropriate toxin binder in the ration of dairy animals. Various types of toxin binders are available in the market (Kolosova and Stroka, 2012). The main characteristic of these toxin binders is its capacity to exchange ions and reduce the aflatoxin's bioavailability to the dairy animals (Karnland et al., 2006). Kaolinite, smectite, chlorites, and micas are groups of silicates or clay-based materials that are used as toxin binders. Montmorillonites are a class of the smectite clay group, which has 3-layer structures that allow for internal adsorption of mono- and divalent ions into each interlayer sheet. Smectites have a wide range of commercial uses and have been reported to adsorb heavy metals, bacteria, and toxic antinutritive agents, such as aflatoxins (Trckova et al., 2014). Bentonite is a composition of aluminosilicate which has a high capacity to adsorb toxins such as aflatoxins and other substances (Magnoli et al., 2010). A number of *in vivo* studies have shown that clay based toxin binders have their ability to reduce $AFM_1$ excretion into milk (Sulzberger et al., 2017).

In view of the above, a study was conducted to evaluate the effect of supplementing sodium bentonite on dry matter intake, milk yield, excretion of $AFM_1$ and blood mineral profile in dairy animals under field condition.

## MATERIALS AND METHODS

### Experimental animals and feeding

A field study was conducted on 30 multiparous lactating animals (26 crossbred cows and 4 buffaloes), selected from four private dairy farms at Varvadiya village in Banaskantha district of Gujarat state. Study was conducted for 60 days during November to December, 2022. To observe the effect of feeding sodium bentonite on $AFM_1$ and its transfer rate from feed to milk, naturally contaminated maize bhardo (ground maize; private brand) was fed to dairy animals during the study period. The animals were divided into three equal groups:Control;Treatment-1 (T1) and Treatment-2 (T2) based on milk yield, fat percent and stage of lactation (90-180 days post calving). Each animal was fed on a basal ration, comprising of 3-4 kg cattle feed, 2-3kg maize bhardo (ground maize grain), 15-20 kg Hybrid Napier green fodder and 4-5kg jowar straw per day. Mineral mixture (Ca-20.0%, P-14.0%) was supplemented @ 100 g/animal/day to all animals during trial period. The feeding was done twice daily in the morning and evening. In addition to basal ration, animals in T1 and T2 groups were supplemented @ 100 g and 150 g commercially available sodium bentonite (Al2O3 - 8.7%, SiO2 – 45.3%, Fe2O3 – 37.3%), respectively per animal per day. Commercial sodium bentonite was procured @ Rs. 6.0/kg, thus the supplemental cost of commercial bentonite clay was less than Rs. 1.0/ animal/day.

### Analytical procedures

Dietary ingredients were analysed for chemical composition using AOAC method (AOAC, 2005). Samples of feeds and fodder were collected at fortnightly intervals for estimation of $AFB_1$ by AOAC 2003.02 method using HPLC (Shimadzu make; model LC-20AD. Level of montmorillonite in bentonite was estimated as per IS 12446 (2007), while mineralogical analysis of bentonite was carried out using X-Ray diffraction (XRD) technique based on EN 13925 (2005) method.

All animals were milked twice daily at 05:00 and 19:00 hrs and milk weights were recorded. Two milk samples were collected from a.m. and p.m. milking on day 0, 15, 30, 45 and 60 during the trial period. After proper mixing, pooled milk samples of 150 ml from each animal was collected and analysed for $AFM_1$ using HPLC method. Excretion of $AFM_1$ was calculated as described by Maki et al. (2016a): Excretion (ìg/day) = concentration of $AFM_1$ in milk on day 60 (ìg/kg) × milk yield on day 60 (kg), and aflatoxin transfer (%) = [$AFM_1$ excretion (ìg/day)/ $AFB_1$ intake (ìg/day)] × 100.

Blood samples (10 ml) were collected before (0 day) and after (60 days) feeding sodium bentonite from the jugular vein into vacutainer tubes, stored in ice during transport, centrifuged at 2,500 rpm for 20 min at 4°C to separate serum and stored at -20°C until analyzed. Serum samples were analysed for macro and micro minerals using Inductively Coupled Plasma-Optical Emission Spectrometer (Optima 3300 RL, Perkin Elmer, Waltham, MA, USA).

### Statistical analysis

Data were analysed using one-way ANOVA with SPSS package programme (SPSS 9.0 software for Window, SPSS Inc., Chicago, IL) as per Snedecor and Cochran (1994).

## RESULTS AND DISCUSSION

### Dry matter intake and milk yield

Chemical composition of feeds and fodder fed to dairy animals during the trial period is given in Table 1. Initial dry matter intake (DMI) in all three groups were similar (11.4 *vs* 11.5 *vs* 11.2 kg; P>0.05). Even after 60 days of feeding sodium bentonite, DMI did not affect amongst the groups (10.9 *vs* 10.7 *vs* 10.5 kg; P>0.05) (Table 3). Although there was numerical reduction in DMI after 60 days, DMI in bentonite supplemented groups was not significantly different (P>0.05) as compared to control group which indicated that supplementation of bentonite had no impact on DMI in experimental animals. Bentonite supplementation also did not affect (P>0.05) milk yield (9.33 *vs* 9.60 *vs* 9.17 kg; P>0.05), fat (5.01 *vs* 4.84 *vs* 5.17%; P>0.05) and protein (3.62 *vs* 3.37 *vs* 3.35; P>0.05) contents in our study.

Table 1. Chemical composition (% DM basis) of feeds and fodder

| Particular | Cattle feed | Maize bhardo | Hybrid napier | Jowar straw |
|---|---|---|---|---|
| Crude protein (%) | 20.7 | 9.89 | 9.32 | 3.26 |
| Ether extract (%) | 3.23 | 3.84 | 1.97 | 1.07 |
| Crude fibre (%) | 11.8 | 5.65 | 30.7 | 28.4 |
| AIA (%) | 2.82 | 0.25 | 2.18 | 4.8 |
| Calcium (%) | 0.86 | 0.06 | 0.28 | 0.48 |
| Phosphorus (%) | 0.55 | 0.47 | 0.30 | 0.14 |
| Aflatoxin $B_1$ (ppb) | 79.1 | 456.9 | BLQ | BLQ |

BLQ = Below Limit of Quantification

Kutz et al. (2009) reported no changes in DMI, milk yield, fat and protein contents when 112 µg of $AFB_1$/kg of total mixed ration (TMR, DM basis) was fed to dairy cows.Stroud (2006) also reported no changes in milk yield due to feeding of 170 µgof $AFB_1$/kg of TMR DM, but the toxin decreased DMI by 1.5 kg of DM/day compared with the DMI of cows not supplemented with $AFB_1$. Various studies have reported no changes in DMI or milk yield when feeding clay based toxin binders to dairy animals (Queiroz et al., 2012; Maki et al., 2016a,b).

### *In vitro* net binding capacity

Clay based toxin binders have been shown to decrease aflatoxin excretion and aflatoxin transfer from feed to milk (Kissell et al., 2013; Barrientos-Velazquez et al., 2016; Maki et al., 2016a). European Union (EU) has approved bentonite as substance for reduction of $AFB_1$ in feed for ruminants. As per the EU specifications, bentonite should contains e”70% montmorillonite and >90% *in vitro* $AFB_1$ binding capacity (EU No. 1060/2013). In our study, commercial sodium bentonite contained 87.7% montmorillonite and 97.6% *in vitro* net binding capacity for $AFB_1$. Bentonite was characterized using XRD technique and the composition of sodium bentonite is given in Table 2.

Table 2. Characterization of sodium bentonite clay (n=1)

| Formula | Wt % |
|---|---|
| $Al_2O_3$ | 8.70 |
| $SiO_2$ | 45.2 |
| $Fe_2O_3$ | 37.2 |
| $Na_2O$ | 1.72 |
| MgO | 1.76 |
| $SO_3$ | 1.29 |
| CaO | 0.84 |
| $TiO_2$ | 1.00 |
| MnO | 0.19 |

Sodium bentonite have a greater water holding capacity than do calcium bentonites. This may affect aflatoxin binding by increasing the surface area. Additionally, the sodium: calcium ratio may play an important role as a source of exchangeable cations, which may form bonds with functional groups in the aflatoxin molecule.

**Feed aflatoxin $B_1$**

Average intake of $AFB_1$ by dairy animals during the study period was 83.1, 89.4 and 105.9 μg/kg DMI (Table 3). Maize bhardo purchased from the private vendor for feeding to dairy animals was highly contaminated with $AFB_1$ and thus it was a major source of $AFM_1$ in milk. The European Food Safety Authority reported that the clinical signs in cattle occurred after exposure of aflatoxins to concentrations of 1500-2200 μg/kg feed (EFSA, 2004).

Table 3. Effect of feeding sodium bentonite on feed intake, milk production and excretion of aflatoxin M1 in milk

| Particular | Control | T1 | T2 |
|---|---|---|---|
| Baseline level (Before feeding toxin binder; 0 day) | | | |
| Dry matter intake (kg/d) | 11.4 ± 0.62 | 11.5 ± 0.51 | 11.3 ± 0.83 |
| Milk yield (kg/d) | 11.0 ± 1.45 | 11.4 ± 1.37 | 10.7 ± 1.95 |
| Fat (%) | 4.82 ± 0.17 | 4.91 ± 0.13 | 5.09 ± 0.15 |
| Protein (%) | 3.51 ± 0.11 | 3.43 ± 0.08 | 3.28 ± 0.10 |
| Intake of AFB1(μg/kg DMI) | 86.9 ± 13.63 | 94.3 ± 12.29 | 114.2 ± 9.62 |
| Excretion of AFM1 in milk (ppb) | 1.39 ± 0.16 | 1.47 ± 0.23 | 1.63 ± 0.25 |
| Daily excretion of AFM1 in milk (μg) | 16.1 ± 4.09 | 18.6 ± 3.95 | 18.8 ± 4.32 |
| Transfer rate from AFB1 to AFM1 (%) | 1.78 ± 0.52 | 1.72 ± 0.37 | 1.37 ± 0.27 |
| 60 days after feeding toxin binder | | | |
| Dry matter intake (kg/d) | 10.9 ± 0.40 | 10.7 ± 0.32 | 10.5 ± 0.49 |
| Milk yield (kg/d) | 9.33 ± 1.37 | 9.60 ± 1.12 | 9.17 ± 1.41 |
| Fat (%) | 5.01 ± 0.17 | 4.84 ± 0.13 | 5.17 ± 0.17 |
| Protein (%) | 3.62 ± 0.10 | 3.37 ± 0.09 | 3.35 ± 0.12 |
| Intake of AFB1(μg/kg DMI) | 83.1 ± 11.01 | 89.4 ± 7.71 | 105.9 ± 9.25 |
| Excretion of AFM1 in milk (ppb) | $1.90^{a}$ ± 0.62 | $0.73^{b}$ ± 0.05 | $0.87^{b}$ ± 0.17 |
| Daily excretion of AFM1 in milk (μg) | $17.46^{a}$ ± 5.66 | $7.12^{b}$ ± 0.85 | $8.42^{b}$ ± 2.71 |
| Transfer rate from AFB1 to AFM1 (%) | $1.66^{a}$ ± 0.39 | $0.80^{b}$ ± 0.06 | $0.73^{b}$ ± 0.18 |

$^{a,b}$Values with different superscripts in a row differ significantly ($P<0.05$).
Control = No feeding of sodium bentonite; T1 = Feeding 100 g sodium bentonite; T2 = Feeding 150 g sodium bentonite.

**Milk aflatoxin $M_1$**

The average daily milk yield were 9.33, 9.60 and 9.17 kg in control, T1 and T2 groups, respectively. Average excretion of $AFM_1$ was 1.90, 0.73 and 0.87 ppb whereas,total daily excretion of $AFM_1$ in milk were 17.46, 7.12 and 8.42 μg in control, T1 and T2 groups, respectively. In our study, level of $AFM_1$ in milk was higher than the maximum permissible limit (0.5 ppb) specified by the FSSAI,which was due to the feeding of contaminated maize bhardo during the study period. Our results indicate that the supplementation of sodium bentonite @ 100 and 150 g in the ration of dairy animals reduced $AFM_1$significantly (P<0.05) by 61.6% and 54.2%, respectively. However, no significant difference (P>0.05) was observed in excretion of $AFM_1$ between the treatment groups (Table 3).

Studies have reported that aflatoxin binders reduce $AFM_1$ concentration in milk. Maki et al. (2016a) used a clay feed additive @ 0.5 and 1.0% of dietary DM and found that both percentages decreased $AFM_1$ concentration in milk (51.3 and 69.7%, respectively). Diaz et al.(2004) compared effects of adding activated carbon, esterified glucomannan, calcium bentonite, and three sodium bentonite products @ 1.2% of diet DM on concentrations of $AFM_1$ in milk of cows fed diets contaminated with 100 µg of $AFB_1$/kg. Respective reductions in milk $AFM_1$ concentrations were 5.4, 59, 31, 65, 50, and 61%.Kutz et al. (2009) also evaluated the effect of adding two hydrated sodium-calcium aluminosilicates or an esterified glucomannan product at 0.5% of diet DM on concentrations of $AFM_1$ in milk of cows fed 100 µg of $AFB_1$/kg of diet. The aluminosilicate products reduced milk $AFM_1$ concentration by 45 and 48%; however, the esterified glucomannan caused a reduction of only 4% of $AFM_1$.

**Transfer of aflatoxin from feed to milk**

Average transfer rate of $AFB_1$from feed to $AFM_1$ in milk of animalsin experimental groups T1 and T2 were 0.80% and 0.73%, respectively, as compared to control group (1.66%).In our study, transmission of aflatoxinfrom feed to milk reduced significantly (P<0.05) by 48.2% and 43.9% in animals fed 100 and 150 g sodium bentonite, respectively. However, non-significant difference (0.80 *vs*0.73; P>0.05) was observed in transmission rate between the treatment groups (Table 3). Because of the low molecular weight of $AFB_1$ and $AFM_1$, the toxins are rapidly adsorbed through membranes by passive mechanism. Upon adsorption, the body's ability to $AFB_1$ detoxification is associated with the action of liver microsomal cytochrome P-450 enzyme and the enzyme S–glutathione transferase. The reduced levels of $AFM_1$ may be due to a strong bond found between toxin binder and aflatoxin in goats (Smith et al.,1994).The transfer rate of dietary aflatoxin into milk was reported between 1 and 6% by the European Food Safety Authority (EFSA, 2004).Garg et al. (2004) reported average transfer rate of $AFB_1$ to $AFM_1$@ 2.55% (1.7-3.9%) in dairy animals of India. Unusan (2006) reported that approximately 0.3-6.2% of $AFB_1$ isconverted into metabolized $AFM_1$ and excreted in milk, dependingon factors such as the genetics of the animals, seasonal variation,the milking process and the environmental conditions.Stroud (2006) reviewed 14 studies on effects of toxin binders on aflatoxin transfer and reported that the mean carryover was about 1.0%, when diets were dosed with up to 150 µg of aflatoxin per kg.

Sherasia et al. (2022) reported that supplementation of commercial bentonite clay @ 150 g/animal/day reduced excretion of $AFM_1$ in milk by 47% in crossbred cows, without affecting DMI (9.08 vs 9.07 kg/day) and milk yield (9.13 vs 8.65 kg/day). The transmission rate of $AFM_1$ from feed to milk was significantly lower (0.75%), as compared to un-supplemented control group (1.17%). Diaz et al. (2004) also reported the reduction of $AFM_1$ by 50-65% in milk while supplementing bentonite @ 1.2% of the dietary dry matter in cow. The transmission rate of $AFM_1$ from feed to milk was lower (0.95-1.13%) as compared to the un-supplemented control group (2.25%). Thus, it can be concluded that the use of sodium bentonite as toxin binder in the ration of dairy animals helps reducing level of $AFM_1$ in milk. The data of the present study indicated that the interaction of toxin binder and aflatoxin provides a protective mechanism against aflatoxin exposure. This is based on the reduced level of $AFM_1$ excretion when toxin binder is supplemented in the aflatoxin contaminated feed, thus suggesting that the bioavailability of aflatoxin is reduced by toxin binder.

**Bioavailability of macro and micro minerals**

Even though clays have been reported to decrease aflatoxins, utilization of certain minerals and vitamins (A, D, and E) have been reported to decrease in the presence of smectite clays (Gowda et al., 2007; Tang et al., 2009; Barrientos-Velazquez et al., 2016). In the present study, serum mineral profiles of experimental animals revealed that the supplementation of sodium bentonite @ 100 and 150 g per animal per day had no significant effect on bioavailability of macro and micro minerals (Table 4), suggesting that sodium bentonite did not alter blood mineral concentrations as previously reported in humans, swine, and chickens (Trckova et al., 2014; Fowler et al., 2015).This may be due to the supplementation of 100 g mineral mixture during the trial period. Bosi et al. (2002) also reported that addition of clinoptilolite at 200 g per day to the diet of lactating dairy cows did not effect on mineral contents of blood plasma (Na, K, Zn, and Ca).

Table 4. Effect of feeding sodium bentonite on serum mineral profiles

| Minerals | T1 | | | T2 | | |
|---|---|---|---|---|---|---|
| | Before feeding bentonite | After feeding bentonite | P value | Before feeding bentonite | After feeding bentonite | P value |
| Calcium (mg/100 ml) | 7.38 | 9.75 | 0.050 | 7.77 | 7.95 | 0.844 |
| Magnesium (mg/100 ml) | 3.42 | 2.76 | 0.485 | 2.90 | 3.23 | 0.141 |
| Sodium (ppm) | 3233 | 3099 | 0.285 | 3198 | 3079 | 0.159 |
| Potassium (ppm) | 206.05 | 289.51 | 0.117 | 218.98 | 236.99 | 0.012 |
| Sulphur (ppm) | 1078 | 1045 | 0.524 | 1032 | 1037 | 0.932 |
| Copper (ppm) | 0.50 | 0.55 | 0.769 | 0.52 | 0.63 | 0.066 |
| Manganese (ppm) | 0.24 | 0.24 | NS | 0.24 | 0.24 | NS |
| Zinc (ppm) | 0.76 | 0.40 | 0.220 | 1.23 | 0.62 | 0.091 |
| Iron (ppm) | 2.82 | 1.47 | 0.546 | 1.35 | 1.52 | 0.255 |
| Cobalt (ppm) | 0.24 | 0.24 | NS | 0.24 | 0.24 | NS |
| Molybdenum (ppm) | 0.24 | 0.24 | NS | 0.24 | 0.24 | NS |
| Arsenic (ppm) | 0.02 | 0.02 | NS | 0.02 | 0.02 | NS |
| Lead (ppm) | 0.02 | 0.02 | NS | 0.02 | 0.02 | NS |
| Selenium (ppm) | 0.09 | 0.10 | 0.422 | 0.09 | 0.11 | 0.124 |

NS=Non significant; P<0.05; T1= Feeding 100 g sodium bentonite; T2= Feeding 150 g sodium bentonite

## CONCLUSION

Present study demonstrated that supplementationof 100 g sodium bentonite clay reduced excretion of aflatoxin $M_1$ in milk, without affecting dry matter intake, milk yield, milk composition and mineral bioavailability in dairy animals. Thus, sodium bentonite can be considered as effective and economical toxin binder for reducing transmission of aflatoxins from feed to milk.

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