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Efficacy of sodium bentonite to ameliorate adverse effects of aflatoxin on *in vitro* rumen fermentation of wheat straw

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ABSTRACT

The present experiment was conducted to study the effect of sodium bentonite (SB) in ameliorating the adverse effects of aflatoxin on *in vitro* rumen fermentation of wheat straw. Five treatment groups, viz. T_1: control (wheat straw); T_2: T_1 + 300 ppb aflatoxin B_1 (AFB_1); T_3: T_2 + 0.33% SB; T_4: T_2 + 0.5% SB and T_5: T_2 + 1.0% SB were prepared and incubated *in vitro*. Truly degradable dry matter (TDDM), truly degradable organic matter (TDOM), gas production (GP), microbial biomass production (MBP) and partitioning factor (PF) values in T_1 were higher than those of other treatment groups (T_2 to T_5). TDDM, TDOM, GP, MBP and PF values in T_2 were lower than those of other treatment groups. The values of these parameters improved with increasing concentration of SB. Total volatile fatty acids (TVFA), acetate (A), propionate (P) and butyrate (B) values in T_1 were higher than those of other treatment groups (T_2 to T_5). TVFA and A value between T_2 and T_3 were statistically similar. TVFA, A, P and B values improved with increasing level of SB, however, these values were lower than that of control even at highest inclusion level of SB. A:P ratio among various treatments did not vary significantly. It was concluded that aflatoxin contamination of feed at 300 ppb level significantly affected the *in vitro* rumen fermentation in terms of reduced truly degradable dry matter, truly degradable organic matter, gas production, microbial biomass production, partitioning factor and total volatile fatty acids concentration. Incorporation of sodium bentonite up to 1.0% level to the aflatoxin-contaminated wheat straw, partially ameliorated the adverse effects of aflatoxin on *in vitro* rumen fermentation parameters.

Keywords: Aflatoxin, *In vitro*, Rumen fermentation, Sodium bentonite**,** Wheat straw

Aflatoxins, produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, are recognized as the most hazardous mycotoxins. The tropical and subtropical climate with hot and humid conditions prevailing in India, coupled with improper harvesting of crops, handling and processing, inadequate drying and storage facilities, and insect infestation make feedstuff susceptible to fungal contamination and mycotoxin production. Presence of aflatoxins in feed is one of the major constraints in maintaining feed quality, because the aflatoxins are widely present in feedstuffs around the world. Liver is the primary target organ for aflatoxins. Long-term intake of aflatoxin contaminated feeds resulted in negative effects on the liver, such as hepatic cells and tissue injury, as well as gross and microscopic abnormalities (Gholami-Ahangaran *et al*. 2016, Singh 2019a). Numerous traditional physical and chemical strategies for the elimination or inactivation of mycotoxins have been reported in the literature (Stoev 2013). Nevertheless, these methods have some limitations concerning safety issues, losses in the nutritional value and the palatability of feeds, coupled with limited efficacy and cost implication. In recent years, using mycotoxin-adsorbing agents to bind mycotoxins in gastrointestinal tract of animals and then decreasing their bioavailability and toxicity, shows a promising potential in feed industrial applications. However, there are various kinds of mycotoxin adsorbing agents and their efficacy in preventing mycotoxicosis varies (Mohamed 2011, Silambarasan *et al*. 2013). *In vitro* studies have shown that SB is strong binder of AFB1 (Veldman *et al*. 1992, Rosa *et al.* 2001). SB and a synthetic zeolite mixture (80:20 ratio) do not depress feed intake or apparent nutrient digestibility (Rizzi *et al*. 1995), but prevent AFB_1 accumulation in the liver of growing lambs and decrease the AFB_1 excretion in urine by several folds (Zaghini *et al*. 1993). Diaz *et al*. (2004) studied the efficacy of several clay types to reduce aflatoxin residues in cows' milk. Also, SB reduced more milk aflatoxin than a similar amount of calcium bentonite. Thus, the objective of the present investigation was to study the effect of SB in ameliorating the adverse effects of aflatoxin on *in vitro* rumen fermentation of wheat straw.

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MATERIALS AND METHODS

Production and analysis of aflatoxin: Aflatoxin was

produced using the fungal strain *Aspergillus flavus* NRRL 6513 obtained from US Department of Agriculture, Illinois, USA. To get the fresh spores, the culture was regularly sub-cultured on Potato Dextrose Agar (PDA) medium slants and stored at 5ºC. Aflatoxin was produced on liquid medium as per the method of Singh and Shamsudeen (2008). Aflatoxin contents were finally quantified using UV-Spectrophotometry.

Experimental design and substrate: Wheat straw was ground to pass a 1 mm sieve and used for experimentation. The following dietary treatments were prepared by mixing the required quantity of AFB_1 and SB to get the desired concentration in the feed (Table 1).

Table 1. Experimental groups and treatments

Group	Treatment
T_1	Basal feed (wheat straw)
T_2	T_1 + 300 ppb AFB_1
T_3	T_2 + 0.33% Sodium bentonite
T_4	T_2 + 0.50% Sodium bentonite
T_5	T_2 + 1.0% Sodium bentonite

Collection of rumen liquor: Fistulated male buffalo, about 5 years-old having 450 kg body weight, fitted with permanent rumen cannula was used as donor animal for collection of rumen liquor. The animal was fed a basal diet of wheat straw offered *ad lib.* and a standard concentrate mixture containing 20% CP and 70% TDN to meet the nutrient requirement for maintenance. The animal was given free access to clean drinking water. Approximately 300 ml of rumen liquor was collected from different depths and directions of reticulo rumen and transferred into pre-heated thermos flask, strained through a four-fold muslin cloth and flushed with CO_2. Rumen liquor was collected in the morning before feeding and watering of the animal as per standard procedure. Rumen fluid–medium mixture (inoculum) was prepared under continuous flushing with CO_2 to maintain anaerobic condition.

In vitro *incubation of substrate and gas production:* Wheat straw (200 mg dry weight) as substrate was weighed into 100 ml calibrated glass syringes and incubated with 30 ml of mixed rumen inoculum at 39ºC for 24 h with parallel incubation of blanks (Menke *et al*. 1979, Menke and Steingass 1988). Each treatment was incubated in triplicate. The syringes were regularly shaken by hand during the incubation period for proper mixing of feeds with rumen inoculum. After 24 h of incubation period, the gas production was recorded by the displacement of piston during incubation period for test substrate and blank syringes. The net gas produced due to fermentation of substrate was calculated by subtracting the value of gas produced in blank syringes from that of test substrates.

In vitro *dry matter degradability and microbial protein synthesis:* After 24 h of incubation period, the content of the syringes, which was extracted in 100 ml of neutral detergent solution (NDS) by boiling for 1 h, was then transferred to 500 ml sproutless beaker followed by filtration on pre-weighed gooch crucibles (G1), washed in hot distilled water and acetone to recover true undigested residue as per the method of Van Soest *et al*. (1991). Crucibles with undigested residue were dried at 100ºC overnight and weighed to determine true undigested residue. Residue was ashed at 500ºC for 3 h to determine true undigested OM, which was corrected for the appropriate blanks. The TDOM was calculated as the difference between OM incubated and the undigested OM recovered in the residue of ND extraction. Truly degradable dry matter (TDDM) and truly degradable organic matter (TDOM) were estimated, and microbial biomass production (MBP) and partitioning factor (PF) were calculated as per the method of Blummel *et al*. (1997).

Microbial biomass production (MBP) = Substrate truly degraded - (gas volume × stoichiometrical factor)

For roughages, the stoichiometrical factor was 2.20.

Estimation of volatile fatty acid: After 24 h incubation, 1 ml of the supernatant of each syringe content was taken in a micro-centrifuge tube containing 0.20 ml metaphosphoric acid (25%, v/v). The mixture was allowed to stand for 2 h at room temperature and centrifuged at 5000×g for 10 min to get a clear supernatant. The supernatant (1 µl) was injected into gas chromatograph equipped with flame ionization detector (FID) and glass column packed with chromosorb as per the method described by Cottyn and Boucque (1968).

Statistical analysis: The data were statistically analyzed using SPSS (20.0 version) following one way analysis. All the observations were recorded at 95% ($P<0.05$) level of significance.

RESULTS AND DISCUSSION

Efficacy of sodium bentonite in ameliorating adverse effects of aflatoxin during in vitro *rumen fermentation*: The data pertaining to TDDM, TDOM, GP, MBP and PF as influenced by various dietary treatments is presented in Table 2, and those of VFAs production is presented in Table 3.

Truly degradable dry matter (TDDM) and truly degradable organic matter (TDOM): The TDDM and TDOM values of T_1 was higher ($P<0.05$) than that of T_2; and those of T_2 were lower ($P<0.05$) than that of T_3, T_4 and T_5 (Table 2). The results revealed that contamination of 300 ppb aflatoxin in feed significantly ($P<0.05$) decreased the DM and OM degradability compared to that of control (T_1). This result was in agreement with that of Singh *et al*. (2020) who also reported reduced TDDM and TDOM in a buffalo diet when the diet was contaminated with 100 to 300 ppb aflatoxin. Similar results were also reported by Westlake *et al*. (1989) wherein IVDMD of alfalfa hay was reduced by 50% with inclusion of 1 µg/ml AFB_1. Also, Mojtahedi *et al*. (2013) reported that IVDMD decreased ($P<0.05$) with inclusion of AFB_1 in culture medium, so that the lowest and the highest IVDMD values were observed in treatments with 900 and 0 ng/ml AFB_1, respectively (0.54 vs. 0.68). Decreased IVDMD with AFB_1 addition can be attributed to

Table 2. Effect of aflatoxin and sodium bentonite on *in vitro* rumen fermentation parameters

Treatment	TDDM (%)	TDOM (%)	GP (ml/g DM)	MBP (mg/100 mg DDM)	PF
T_1	40.85±0.08^e	41.23±0.08^e	148.98±0.16^d	20.70±0.20^c	2.73±0.01^e
T_2	36.02±0.12^a	37.11±0.16^a	140.25±0.32^a	17.37±0.47^a	2.56±0.01^a
T_3	37.04±0.09^b	38.28±0.20^b	142.51±0.22^b	18.70±0.41^b	2.60±0.01^b
T_4	37.95±0.07^c	38.80±0.11^c	144.44±0.19^c	18.48±0.32^b	2.62±0.01^c
T_5	38.92±0.15^d	39.40±0.14^d	144.94±0.20^c	19.29±0.35^b	2.68±0.01^d

Values bearing different superscripts in a column differ significantly (P<0.05).

compromised ruminal function by reducing fibre digestion and VFA production (Fehr and Delage 1970, Helferich *et al*. 1986a, b). However, some studies reported no effect of AFB_1 level on *in vitro* dry matter disappearance of feed substrate (Kiessling *et al*. 1984, Jiang *et al*. 2012). Yeanpet *et al*. (2018) also found that the IVDMD and IVOMD were not significantly affected by AFB_1. In the present study, inclusion of SB to 300 ppb aflatoxin contaminated feed (T_3 to T_5) significantly (P<0.05) ameliorated the adverse effects of aflatoxin on the TDDM and TDOM in a dose dependent manner. However, addition of SB in aflatoxin contaminated feed even at highest level (1.0%) (T_5) could not reverse the TDDM and TDOM value equivalent to that of control (T_1). Ameliorative effects of SB on adverse effects of aflatoxin are also reported in literature. Rosa *et al*. (2001) reported a SB from South Argentina showing high ability of sequestering AFB_1 from aqueous solution. A commercial SB adsorbent at 0.3% level was used to prevent the effect of aflatoxin (50 µg of AFB_1/ kg of feed) on broiler productivity, biochemical parameters, macroscopic and microscopic liver changes and AFB_1 liver residues (Magnoli *et al*. 2011). Chicks receiving 100 µg/kg aflatoxin contaminated diets had suppressed body weight, feed consumption and FCR value, which were significantly improved with the addition of 0.5% SB (Pasha *et al.* 2007). Safaeikatouli *et al*. (2010) reported that addition of SB increased the total protein value equal to control in broiler chickens. Silambarasan *et al*. (2013, 2015, 2016) reported that diatomaceous earth, sodium bentonite and zeolite either at 0.5% or 1% level were partially to completely effective in ameliorating the adverse effects of aflatoxin in broiler chickens. Among three mycotoxin adsorbents tested, diatomaceous earth was least effective in comparison to sodium bentonite and zeolite. However, combination of the binders at a time was most effective in ameliorating the adverse effects of aflatoxin B_1 in broiler chickens. The present study further revealed that inclusion of SB at any level to the 300 ppb aflatoxin-contaminated feed, partially ameliorated the ill effects of aflatoxin on DM and OM degradability.

Gas production and microbial biomass production: The gas production (GP) value in T_1 was higher (P<0.05) than that of T_2. The GP value between T_4 and T_5 was statistically similar (Table 2). The results revealed that aflatoxin contamination of feed at 300 ppb level decreased (P<0.05) the GP compared to that of control (T_1). This result was in agreement with Singh *et al*. (2020) who also reported reduced GP when the diet was contaminated with 100 to 300 ppb AFB_1. Also, Mojtahedi *et al*. (2013) reported that by increasing the level of AFB_1 from 0 to 900 ng/ml, the GP rate decreased from 0.071 to 0.051 and cumulative GP decreased from 196.4 to 166.0 ml/g DM, respectively. Similarly, Jiang *et al*. (2012) and Helferich *et al*. (1986a, b) also observed similar results of reduced gas production parameters when AFB_1 was added. These depressions in the GP suggested that microbial populations were altered by AFB_1 contamination of feed. Thus, the inclusion of SB to the 300 ppb aflatoxin contaminated feed significantly (P<0.05) alleviated the adverse effects of aflatoxin on GP in a dose dependent manner, however, even at the highest level (1.0%) of SB (T_5), the GP value was lower (P<0.05) than that of control (T_1), With respect to microbial biomass production (MBP), the MBP value in T_1 was higher (P<0.05) than that of T_2. The MBP value among T_3, T_4 and T_5 did not vary significantly. The results of present investigation revealed that aflatoxin contamination of feed at 300 ppb level resulted in significant decrease in the MBP value compared to that of control. This finding was in agreement with that of Singh *et al*. (2020) who also reported significantly reduced MBP due to aflatoxin contamination of feed at 100 to 300 ppb level in the diet of buffalo. The present study further revealed that inclusion of SB at 0.33% level (T_3) to the 300 ppb aflatoxin contaminated feed ameliorated the adverse effects of aflatoxin on MBP.

Table 3. Effect of aflatoxin on volatile fatty acids production

Treatment	TVFA (mM/100ml)	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	A:P ratio
T_1	6.26±0.04^c	4.51±0.09^c	1.27±0.01^d	0.50±0.01^d	3.55±0.08^a
T_2	4.99±0.04^a	3.56±0.02^a	0.90±0.01^a	0.35±0.01^a	2.97±0.96^a
T_3	5.20±0.05^a	3.59±0.04^a	0.97±0.01^b	0.39±0.00^b	3.69±0.04^a
T_4	5.59±0.10^b	4.00±0.05^b	1.07±0.03^c	0.41±0.01^{bc}	3.75±0.11^a
T_5	5.71±0.13^b	4.07±0.03^b	1.09±0.03^c	0.42±0.01^c	3.75±0.12^a

Values bearing different superscripts in a column differ significantly (P<0.05).

Also, the addition of SB level above 0.33% to the aflatoxin contaminated feed did not produce any beneficial effect in ameliorating adverse effects of aflatoxin on MBP.

Partitioning factor (PF): The PF value of T_1 was higher ($P<0.05$) than that of T_2. The PF value of all other treatment groups (T_2 to T_5) was lower ($P<0.05$) than that of T_1. The present investigation revealed that aflatoxin contamination of feed at 300 ppb level resulted in decrease ($P<0.05$) in the PF value compared to that of control. This result was in agreement with that of Singh *et al*. (2020) wherein the buffalo diet was contaminated with 100 to 300 ppb aflatoxin. The present study further revealed that incorporation of SB at 0.33 to 1.0% levels to the 300 ppb aflatoxin contaminated feed significantly ($P<0.05$) ameliorated the adverse effects of aflatoxin on PF value in a dose dependent manner. However, even at the highest level (1.0%) of SB (T_5) inclusion to the aflatoxin contaminated feed, the PF value was lower ($P<0.05$) than that of control (T_1). A feed with higher PF value means that proportionally more of the degraded matter is incorporated into microbial mass, i.e. the efficiency of microbial protein synthesis is higher. Roughages with higher PF have been shown to have higher dry matter intake (Harikrishna *et al*. 2012).

Volatile fatty acids (VFAs) production: The total volatile fatty acids (TVFAs), acetate (A), propionate (P) and butyrate (B) values in T_1 were higher ($P<0.05$) than that of T_2. The TVFA and A value between T_2 and T_3; and between T_4 and T_5 were statistically similar (Table 3). The P and B value of T_2 was lower ($P<0.05$) than those of T_3, T_4 and T_5. The B value between T_3 and T_4; and between T_4 and T_5 was statistically similar. The B value of T_3 was lower ($P<0.05$) than that of T_5. The results revealed that aflatoxin contamination @ 300 ppb in feed significantly decreased the TVFA, A, P, and B production compared to that of control. This finding of reduced VFA due to aflatoxin concentration was in agreement with Jiang *et al*. (2012) and Singh *et al*. (2020) who also reported that the VFA concentration decreased with the increase of AFB_1 level in feed. Cellulose degradation, VFA production, ammonia production, and proteolysis were decreased by AFB_1 at 0.2-0.8 mg/kg body weight in acute bovine aflatoxicosis (Cook *et al*. 1986). Also, the production of VFA irrespective of substrate was inhibited by the increasing dose levels of AFB_1, which was consistent with the reduction in the asymptotic gas volume. The suppression of VFA, gas production and ammonia N implicated that microbial activity was inhibited regardless of substrate used. Contrary to this, Edrington *et al*. (1994) found no differences in ruminal VFA concentrations in growing lambs fed 2.5 mg AFB_1 per kg diet. Helferich *et al*. (1986a) also reported that AFB_1 at 60-600 ppb did not influence the production of VFA in steers. In another experiment, ingestion of 0.714 µmol AFB_1 per animal did not influence the ruminal VFA production in lactating goats (Helferich *et al*. 1986b). With regard to A:P ratio, the A:P ratio value in various treatments (T_1 to T_5) varied from 2.97 (T_2) to 3.75 (T_4 and T_5). The A:P ratio value among various treatment groups (T_1 to T_5) did not vary significantly ($P>0.05$). The present investigation revealed that aflatoxin contamination of feed at 300 ppb level (T_2) did not produce any significant effect on A:P ratio as compared to that of control (T_1), however, the A:P ratio value in aflatoxin contaminated group (T_2) was numerically lower than that of control. Incorporation of SB to the aflatoxin (300 ppb) contaminated feed (T_3 to T_5) did not produce any effect on the A:P ratio compared to that of aflatoxin contaminated group (T_2), however, the A:P ratio of groups T_3 to T_5 was numerically higher than that of (T_2). This finding revealed that aflatoxin (300 ppb) contamination of feed did not change the A:P ratio significantly. However, Singh *et al*. (2020) reported increased A:P ratio value when the bufffalo diet was contaminated with 100 to 300 ppb aflatoxin. Further, the addition of SB to the aflatoxin contaminated feed did not produce any significant effect on the A:P ratio.

It was concluded that aflatoxin contamination of feed at 300 ppb level significantly affected the *in vitro* rumen fermentation in terms of reduced truly degradable dry matter, truly degradable organic matter, gas production, microbial biomass production, partitioning factor and total volatile fatty acids concentration. Incorporation of sodium bentonite up to 1.0% level to the aflatoxin contaminated feed partially ameliorated the adverse effects of aflatoxin on *in vitro* rumen fermentation parameters.

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