RESEARCH ARTICLE

Effectiveness of Hydrated Sodium Calcium Aluminosilicate (HSCAS) in Mitigating the Adverse Effects of Aflatoxin on *In Vitro* Rumen Fermentation of Wheat Straw

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Abstract

To study the effect of hydrated sodium calcium aluminosilicate (HSCAS) in ameliorating adverse effects of aflatoxin on *in vitro* rumen fermentation, five treatment groups, *viz.,* T_1: control (wheat straw; 0.2 g); T_2: T_1+300 ppb Aflatoxin B_1 (AFB_1); T_3: T_2+0.33% HSCAS; T_4: T_2+0.5% HSCAS and T_5: T_2+ 1.0% HSCAS were prepared and incubated *in vitro*. The results revealed that truly degradable dry matter (TDDM), truly degradable organic matter (TDOM), gas production (GP), microbial biomass production (MBP) and partitioning factor (PF) values in aflatoxin contaminated group (T_2) were lower ($p<0.05$) than those of other treatment groups. The TDDM, TDOM, GP, MBP and PF values in control group (T_1) were higher than those of other treatment groups, *i.e.,* T_2 to T_5. These parameters improved with increasing concentration of HSCAS. The total volatile fatty acids (TVFA), acetate (A), propionate (P) and butyrate (B) values in control group (T_1) were higher ($p<0.05$) than those of other treatment groups, *i.e.,* T_2 to T_5. The TVFA, A, P and B values in aflatoxin contaminated T_2 group were lower ($p<0.05$) than those of other treatment groups. The A:P value among various dietary treatments did not vary significantly. It was concluded that aflatoxin contamination of feed (wheat straw) at 300 ppb level significantly affected the *in vitro* rumen fermentation in terms of reduced TDDM, TDOM, GP, MBP, PF, TVFA concentration. Inclusion of hydrated sodium calcium aluminosilicate to the aflatoxin contaminated feed partially ameliorated the adverse effects of aflatoxin on *in vitro* rumen fermentation parameters.

Key words: Aflatoxin, Buffalo, Hydrated sodium calcium aluminosilicate, *In vitro* Rumen fermentation.

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Introduction

Mycotoxin contamination occurs widely in feedstuffs of plant origin, especially in cereals, fruits, almonds, seeds, fodder, and other agricultural feed or food intended for animal or human consumption (Wu *et al*., 2014, 2015). It is also worth noting that human exposure to mycotoxins may be caused by not only consumption of plant-derived foods contaminated with toxins, but also the carry-over of mycotoxins and their metabolites in animal products, such as animal tissues, milk and eggs. Aflatoxins, produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, are recognized as the most hazardous mycotoxins. The liver is the primary target organ for aflatoxin. Long-term intake of feeds contaminated with aflatoxin results in negative effects on the liver, such as hepatic cell and tissue injury, as well as gross and microscopic abnormalities (Gholami-Ahangaran *et al*., 2016). After aflatoxin B_1 is consumed by lactating animals another carcinogenic mycotoxin can be detected in the milk. This is the so-called aflatoxin M_1 or "milk toxin" and consists aflatoxin's B_1 major metabolite. The consumption of even low concentrations of aflatoxin B_1 by animals could lead to the excretion of aflatoxin M_1 into milk in concentrations exceeding the maximum permissible limit set by the European Union (50 ppt) making the milk liable for public health issues (Kourousekos *et al*., 2012). Because avoiding consumption of aflatoxin contaminated foods for many is simply not feasible, effective means for reducing dietary exposure to aflatoxins are highly desirable (Phillips *et al*., 2006).

There are many studies that have demonstrated the capability of clay minerals to adsorb aflatoxin and decrease AFM_1 in milk and alleviate inflammatory suppression. Kutz *et al*. (2009) reported a 46% reduction in aflatoxin excretion

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and a 47% reduction in aflatoxin transfer from feed to milk by feeding a silicate clay mixture known as hydrated sodium calcium aluminosilicates (HSCAS). A similar aluminosilicate product was used by Queiroz *et al*. (2012) and found a 45% reduction in milk AFM_1 as well as a significant improvement to the immune challenge effect of aflatoxin on haptoglobin. HSCAS included at 0.5% to 2.0% of the diet is well documented to adsorb aflatoxin and to prevent aflatoxicosis in various animal species including dairy cows (Ledoux *et al*., 2009) and dairy goats (Smith *et al*., 1994). Responses to HSCAS appear to be dose dependent (Ledoux *et al*., 2009). The objective of the present investigation was to study the ameliorative effects of hydrated sodium calcium aluminosilicate on adverse effects of aflatoxin on *in vitro* rumen fermentation.

Materials and Methods

Production and Analysis of Aflatoxin

Aflatoxin was produced using the fungal strain *Aspergillus flavus* NRRL 6513 that was obtained from U.S. 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 5°C. Aflatoxin was produced on liquid medium as per the method of Singh and Shamsudeen (2008). Aflatoxin content in treated feed was finally quantified using UV-Spectrophotometry.

Experimental Design and Substrate

Feed sample (wheat straw) was ground to pass a 1 mm sieve and used for experimentation. The dietary treatments prepared by mixing the required quantity of aflatoxin B_1 and HSCAS to get their desired concentration in the feed were: T_1: control (wheat straw; 0.2 g); T_2: T_1+300 ppb Aflatoxin B_1 (AFB_1); T_3: T_2+0.33% HSCAS; T_4: T_2+0.5% HSCAS and T_5: T_2+ 1.0% HSCAS.

Collection of Rumen Liquor

Fistulated male buffalo, fitted with permanent rumen cannula, about 2.5 years-old having 350 kg body weight 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 before feeding and watering as per standard procedure and transferred into pre-heated thermos flask, strained through a 4-fold muslin cloth and flushed with CO_2. 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

200 mg dry weight of each feed substrate was weighed into 100 mL calibrated syringes and incubated with 30 mL of mixed rumen inoculum at 39°C for 24 h with parallel incubation of blanks (Jiang *et al.,* 2018). Each substrate 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 was transferred to 500 mL spoutless beakers, which was extracted in 100 mL of neutral detergent solution (NDS) by boiling for 1 h, followed by filtration on pre-weighed gooch crucibles (G1), and washing 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 100°C overnight and weighed to determine true undigested residue. Residue was ashed at 500°C 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) was estimated, and microbial biomass production (MBP) and partitioning factor (PF) was calculated as per the method of Blummel *et al*. (1997).

Microbial biomass production (MBP) = Substrate truly degraded - (gas volume X 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 5,000 × g for 10 min to get 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

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

Results and Discussion

The data pertaining to truly degradable dry matter (TDDM), truly degradable organic matter (TDOM), gas production (GP), microbial biomass production (MBP) and partitioning

factor (PF) as influenced by various dietary treatments are presented in Table 1. The data pertaining to volatile fatty acids (VFA) production are presented in Table 2.

Truly Degradable Dry Matter (TDDM) and Truly Degradable Organic Matter (TDOM)

The TDDM and TDOM values in aflatoxin contaminated group (T_2) were lower ($p<0.05$) than those of other treatment groups. The TDDM and TDOM values in control group (T_1) were higher than those of other treatment groups, *i.e.,* T_2 to T_5. Among the treatment groups, the TDDM and TDOM values gradually and significantly ($p<0.05$) increased from T_2 to T_5 group. The study indicated that inclusion of aflatoxin @ 300 ppb in feed significantly ($p<0.05$) decreased the DM and OM degradability compared to that of control. Similar results were also reported by Mojtahedi *et al*. (2013), wherein IVDMD was reduced significantly ($p<0.05$) with inclusion of AFB_1 in culture medium. Singh *et al*. (2020) also reported reduced TDDM and TDOM of buffalo diet when the diet was contaminated with 100 to 300 ppb aflatoxin. Singh and Saini (2020a,b) also reported reduced TDDM and TDOM of 300 ppb aflatoxin contaminated buffalo diet. Decreased IVDMD with AFB_1 addition can be attributed to compromised ruminal function by reducing fibre digestion and volatile fatty acid production (Helferich *et al*., 1986a,b). However, some studies reported no effect of AFB_1 on *in vitro* dry matter disappearance of hay (Jiang *et al*., 2012). Yeanpet *et al*. (2018) also reported that IVDMD and IVOMD were not significantly affected by AFB_1.

In the present study, inclusion of hydrated sodium calcium aluminosilicate in feed significantly ($p<0.05$) improved the TDDM and TDOM in a dose dependent manner. However, inclusion of HSCAS in feed even at highest level (1.0%) could not reverse the TDDM and TDOM equivalent to that of control (T_1). HSCASs are thought to absorb aflatoxin selectively during the digestive process, which renders much of the aflatoxin unavailable for absorption from the gastrointestinal tract (Kubena *et al*., 1990). The chemisorption of aflatoxin to HSCAS involves the formation of a complex by the β-keto-lactone or bilactone system of aflatoxin with uncoordinated metal ions in HSCAS (Sarr *et al*., 1990). AFB_1 may react at surfaces and within the interlayers of HSCAS particles (Phillips *et al*., 2002, 2008). However, HSCAS is characterized as an "aflatoxin-selective clay" and is not a good adsorbent of other mycotoxins, and therefore, is not expected to be protective against feeds containing multiple mycotoxins. In one of the studies, a total of 80% of AFB_1 could be adsorbed by HSCAS *in vitro* and could prevent aflatoxicosis (Phillips *et al*., 2002).

Gas Production and Microbial Biomass Production

The gas production (GP) value in control group (T_1) was higher ($p<0.05$) than those of T_2 to T_5 treatment groups. The GP value in T_2 group was lower ($p<0.05$) compared to other treatment groups, and it increased gradually and significantly ($p<0.05$) from T_2 to T_5 group. The results indicated that aflatoxin contamination of wheat straw at 300 ppb level significantly ($p<0.05$) decreased the gas production compared to that of control (T_1). The present findings were in agreement with Mojtahedi *et al*. (2013), who reported that by increasing the level of AFB_1 from 0 to 900 ng/mL, the gas production rate decreased from 0.071 to 0.051 and cumulative gas production decreased from 196.4 to 166.0 mL/g DM, respectively. Similarly, Jiang *et al*. (2012) also reported that the gas production parameters were reduced when AFB_1 was added. Singh *et al*. (2020) and Singh and Saini (2020a,b) also reported reduced gas production in a buffalo diet when the diet was contaminated with 100 to 300 ppb aflatoxin. These depressions in the gas production suggest that microbial populations are altered by AFB_1 contamination of feed.

In the present study, inclusion of HSCAS to the aflatoxin contaminated feed significantly ($p<0.05$) ameliorated the adverse effects of aflatoxin on gas production in a dose dependent manner, however, even the highest level (1.0%) of *Saccharomyces cerevisiae* could not reverse the gas production value equivalent to that of control (Karami *et al.,* 2017). With respect to microbial biomass production (MBP), the value in control group (T_1) was higher ($p<0.05$) than those of T_2, T_3 and T_4. The MBP value in T_2 group was lower ($p<0.05$) than those of T_1, and T_3 to T_5 treatment groups. The MBP value of group T_5 was statistically similar to that of control. The results of present investigation revealed that inclusion of aflatoxin to the feed at 300 ppb level resulted in significant decrease in the MBP compared to that of control. Similar results of reduced MBP due to aflatoxin contamination were also reported by Singh *et al*. (2020) and Singh and Saini (2020a,b). Inclusion of HSCAS at the highest level (1.0%) reversed the MBP value equivalent to that of control.

Table 1: Effect of aflatoxin on *in vitro* rumen fermentation parameters

Treatments	TDDM %	TDOM %	GP mL/g DM	MBP mg/100mg DDM	PF
T_1	40.85 ± 0.08^{e}	41.23 ± 0.08^{e}	148.98 ± 0.16^{e}	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	37.48 ± 0.15^{b}	38.56 ± 0.20^{b}	143.06 ± 0.16^{b}	18.91 ± 0.40^{b}	2.62 ± 0.01^{b}
T_4	38.32 ± 0.12^{c}	39.11 ± 0.05^{c}	144.80 ± 0.04^{c}	18.91 ± 0.17^{b}	2.64 ± 0.01^{c}
T_5	39.20 ± 0.12^{d}	39.92 ± 0.03^{d}	145.78 ± 0.10^{d}	20.03 ± 0.10^{c}	2.69 ± 0.01^{d}

Values bearing different superscripts within a column differ significantly ($p<0.05$).

Table 2: Effect of aflatoxin on volatile fatty acids production during *in vitro* rumen fermentation

Treatments	TVFA mM/ 100 mL	Acetate mM/ 100 mL	Propionate mM/ 100 mL	Butyrate mM/ 100 mL	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	5.23±0.04^{a}	3.64±0.03^{a}	0.98±0.01^{b}	0.40±0.00^{b}	3.69±0.03^{a}
T_4	5.62±0.11^{b}	4.06±0.03^{b}	1.08±0.02^{c}	0.42±0.00^{bc}	3.75±0.10^{a}
T_5	5.82±0.14^{b}	4.14±0.02^{b}	1.11±2.02^{c}	0.43±0.01^{c}	3.73±0.08^{a}

Values bearing different superscripts within a column differ significantly ($p<0.05$).

Partitioning Factor (PF)

The partitioning factor (PF) value in control group (T_1) was higher ($p<0.05$) than those of all treatment groups. The PF value in aflatoxin contaminated group (T_2) was lowest and it increased gradually and significantly ($p<0.05$) from T_2 to T_5 groups. The results revealed that inclusion of aflatoxin to the feed at 300 ppb level resulted in significant decrease in the PF value compared to that of control. Singh *et al*. (2020) and Singh and Saini (2020a,b) also reported reduced partitioning factor in a buffalo diet when the diet was contaminated with 100 to 300 ppb aflatoxin. In the present study, inclusion of HSCAS in feed significantly ($p<0.05$) improved the PF value in a dose dependent manner. However, inclusion of HSCAS in feed even at highest level (1.0%) could not reverse the PF value equivalent to 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 (TVFA), acetate (A), propionate (P) and butyrate (B) values in control group (T_1) were higher ($p<0.05$) than those of other treatment groups, *i.e.,* T_2 to T_5. The TVFA, A, P and B values in aflatoxin contaminated T_2 group were lower ($p<0.05$) than those of other treatment groups. The TVFA value in T_3 was lower ($p<0.05$) than that of T_4. The TVFA values between groups T_2 and T_3; and between T_4 and T_5 did not vary significantly. The A and P value of T_3 was lower ($p<0.05$) than those of T_4 and T_5. The A and P values between groups T_4 and T_5 did not vary significantly. The B value in group T_3 was lower ($p<0.05$) than that of T_5. The B value between groups T_3 and T_4; and between T_4 and T_5 did not vary significantly. The A:P ratio in control group (T_1) was numerically higher than that of aflatoxin contaminated group (T_2). The A:P value among various dietary treatments (T_1 to T_5) did not vary significantly. The results of the present study revealed that inclusion of aflatoxin @ 300 ppb in feed significantly decreased the TVFA, A, P, and B production compared to that of control.

Present finding of reduced VFA due to aflatoxin concentration was in agreement with Jiang *et al*. (2012), Singh *et al*. (2020), and Singh and Saini (2020a,b), who also reported that the VFA concentration decreased with the increase of AFB_1 dose level. 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). In the present study, inclusion of HSCAS to the aflatoxin contaminated feed partially ameliorated the adverse effects of aflatoxin on VFA production in a dose dependent manner. However, inclusion of aflatoxin alone or HSCAS to the aflatoxin (300 ppb) contaminated feed did not produce any significant effect on A:P ratio.

Conclusion

The study examined the impact of aflatoxin contamination on *in vitro* rumen fermentation parameters, focusing on truly degradable dry matter (TDDM), truly degradable organic matter (TDOM), gas production (GP), microbial biomass production (MBP), partitioning factor (PF), and volatile fatty acids (VFA) production. It concludes that aflatoxin contamination at 300 ppb substantially hinders rumen fermentation, reducing nutrient degradability, microbial activity, and VFA production. Although HSCAS partially mitigates these adverse effects, it does not fully counteract aflatoxin's impact, indicating the need for comprehensive strategies to manage aflatoxin contamination in ruminant diets.

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