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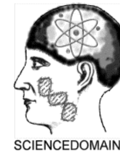

Effect of Aflatoxin B1 on *in vitro* Rumen Microbial Fermentation Responses Using Batch Culture

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Authors' contributions

This work was carried out in collaboration between all authors and all authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To evaluate the effect of aflatoxin B1 (AFB1) on *in vitro* dry matter disappearance (IVDMD), gas production and ammonia-N formation of an alfalfa hay based diet using batch culture system.
Place and Duration of Study: Department of Animal Science, between July 2011 and August 2012.
Methodology: In an anaerobic batch culture system, 50 ml of buffered rumen fluid was dispensed into a 125-ml serum bottle containing 0.5 g dry matter (DM) of the experimental diet. Experimental treatments included four dose levels of AFB1 (0, 300, 600 and 900 ng/ml). All bottles were purged with anaerobic CO2, sealed and placed in a shaking water bath for 72 h at 38.6ºC. Gas production of each bottle was recorded at 2, 4, 8, 12, 16, 24, 48 and 72 h of the incubation and then gas released. The batch cultures were repeated in three incubation runs. After 72 h incubation, bottles were opened and 2-ml sample of each bottle were taken for ammonia-N analysis. The biomass residues were centrifuged and the pellet was dried at 65℃ for the determination of t he residual DM and IVDMD.
Results: Addition of AFB1 affected the rate and cumulative gas production ($P<0.05$), so,

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by increasing the level of AFB1 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. In addition, IVDMD decreased significantly with inclusion of AFB1 in culture medium, so that the lowest and the highest IVDMD values were observed in treatments with 900 and 0 ng/ml AFB1, respectively (0.54 vs. 0.68). The results indicated that addition of AFB1 significantly ($P<0.05$) decreased ammonia-N concentrations, so the lowest value was observed at 900 ng/ml AFB1.

Conclusion: The addition of different levels of AFB1 affected *in vitro* fermentation characteristic, as represented in reduced gas production, dry matter digestibility and ammonia-N concentrations. Therefore it is necessary to control and manage aflatoxin contaminations in ruminants.

Keywords: Aflatoxin B1; rumen fermentation; batch culture.

1. INTRODUCTION

Aflatoxins (AF) are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 (AFB1), the most abundant AF in naturally contaminated foods and feeds, is toxic and carcinogenic to humans and animals [1]. This toxin becomes stable once formed in grain, resistant to degradation during normal milling and storage [2]. This presents the toxicity of contaminated feedstuffs as a significant, potential health hazard to animals and human beings. In ruminants, toxic effects are associated with liver damage, diminished growth efficiency, diminished milk production and quality and impaired resistance to infectious diseases [3]. In other hand, rumen motility was decreased after aflatoxin administration in steers at the dose rates of 200-800 µg of AFB1 per kg diet [4]. Fehr and Delage [5] observed that levels of aflatoxin greater than 200 ng per ml in an *in vitro* artificial rumen system decreased cellulolysis and ammonia-N formation, but some studies reported no effect of AFB1 on *in vitro* dry matter disappearance of hay [6,7].

Although it appears that some microorganisms in the rumen may be disturbed by aflatoxin, the relative aflatoxicosis resistance of ruminant animals in comparison to nonruminants, suggest that other rumen microorganisms may be able to degrade and transform aflatoxin to less toxic metabolites (e.g., aflatoxicol). [7,8,9,10]. It has shown that the carbonyl group of the cyclopentane ring of aflatoxin Bl was reduced to form aflatoxicol [11], which is 18 times less toxic than aflatoxin B1.

In recent years some studies have shown that adsorbent products including clays (typically hydrated sodium calcium aluminosilicates), activated carbons, and yeast products are effective in sequestering and binding aflatoxin [12,13,14,15]. These products are capable of attaching aflatoxin to their surface without any chemical action. Bentonite is one of the best known and the most commonly used clay for aflatoxin attachment. Montmorillonite which is a nano-structured and nano-porous member of smectite group is the dominant mineral constituent of a bentonite affecting the whole bentonite properties. However these strategies mainly offer the potential to reduce transfer of aflatoxin from feed to bloodstream and reduce milk aflatoxin residues in ruminants, while, there is no document about preventive effect of adsorbent products on rumen dysfunction caused by feed born aflatoxin. The objective of the present study was to evaluate effect of AFB1 on *in vitro* dry matter disappearance, gas production and ammonia-N formation of an alfalfa hay based diet using batch culture system.

2. MATERIALS AND METHODS

2.1 Experimental Diet and Aflatoxin Preparation

Five mg of AFB1 (Sigma–Aldrich, Catalog number: A6636) was dissolved in 2 ml absolute methanol, then diluted with sterilized deionized water to make 15.3 µg/ml, 30.6 µg/ml, and 45.9µg/ml AFB1 solutions. The experimental diet was a mixture of alfalfa hay and concentrate (Table 1). Samples were oven dried (66ºC, 48 h), then ground to pass through 1.5 mm screen. A sample of 500 mg of the experimental diet was used for batch culture incubation. The experimental diet samples were analyzed for content of DM (method 930.5), crude protein (CP) (method 984.13), neutral detergent fiber (NDF) (method 2002.04) and acid detergent fiber (ADF) (method 973.18) with AOAC methods [16].

Table 1. Ingredients and chemical composition of experimental diet

Item	Amount (% of diet DM)
Ingredients	
Alfalfa hay	50.0
Barley grain, rolled	20.5
Corn grain, grind	17.0
Sugar beet pulp	3.5
Soybean meal	5.5
Canola meal	3.5
Chemical composition	
Crude protein	15.2
Neutral detergent fiber	31.6
Acid detergent fiber	23.0
Non fiber carbohydrates	36.7

2.2 *In vitro* Batch Culture

Ruminal fluid was collected from 4 fistulated steers (620 ± 45 kg body live weight). Animals had free access to water and were fed 10.4 kg total mixed ration divided into two equal meals at 07:00 and 17:00 h. The ration contained [per kg of dry matter (DM)]: 250 g corn silage, 250 g alfalfa hay, 160 g corn grain, 160 g barley grain, 45g wheat bran, 110 g soybean meal, 5.5 g dicalcium phosphate, 4.5 sodium chloride and 5 g commercial vitamin and trace mineral premix (each kg containing: 190g Ca, 90g P, 50g Na, 19g Mg, 3g Cu, 3g Fe, 2g Mn, 3g Zn, 100mg Co, 100mg I, 1mg Se, 500,000 IU vitamin A, 100,000 IU vitamin D3, 100 mg vitamin E, 3g antioxidant). Net energy for growth and CP content were 6.57 MJ and 155 g, per kg DM, respectively. Animals were cared for according to the Iranian Council of Animal Care guidelines. Ruminal fluid was immediately collected before the morning feeding, strained through four layers of cheesecloth to eliminate large feed particles and transferred to the laboratory in a pre-warmed thermos.

Procedure of *in vitro* batch culture was performed according to the Menke and Steingass [17]. In an anaerobic condition, 50 ml of buffered rumen fluid [ratio of buffer to rumen fluid was 2:1]. was dispensed with Pipetor pump into a 125-ml serum bottle containing 0.5 g DM of the experimental diet. Experimental treatments included four dose levels of AFB1 (0, 300, 600 and 900 ng/ml of medium). The respective AFB1 solutions (1 ml) were added to the bottles, resulting in the three dose levels of 300, 600 and 900 ng/ml AFB1, and 1 ml AFB1-

free methanol solution diluted with deionized water was added to the zero dose treatment. All bottles were purged with anaerobic CO2, sealed with rubber stoppers and placed in a shaking water bath for 72 h at 38.6ºC. To prevent accumulation of gas produced, head space gas pressure of each bottle was recorded using a pressure transducer [18] at 2, 4, 8, 12, 16, 24, 48 and 72 h of the incubation and then gas released. The batch cultures were repeated in three incubation runs. After 72 h incubation, bottles were respectively transferred to refrigerator to stop fermentation, and then opened. A 2-ml sample of each filtrate bottle was taken, then acidified with 2-ml of 0.2 N HCl and frozen at -20ºC. The biomass residues were centrifuged at 1000×g for 10 min at 4ºC. The supernatant in each bottle was decanted and the pellet was dried at 65ºC to a constant weight for the determination of the residual DM.

2.3 Calculations and Statistical Procedure

Gas pressure was converted into volume using an experimentally calibrated curve. Data of cumulative gas production data were fitted to the exponential equation $GP=b(1-e^{-Ct})$ [19], where b is the gas production from the fermentable fraction (mL), the gas production rate constant C (mL/h), t the incubation time (h) and GP is the gas produced at time t (ml/g DM).
In vitro DM disappearance (IVDMD) was calculated as the difference between initially incubated DM and residual DM, corrected by blanks. Ammonia-N concentration was determined by a colorimetric method [20].

Data were statistically analyzed using GLM procedure of SAS [21] with flowing statistically model; $y=\mu+T_i+ e_{ij}$, where y= depended variable, μ= overall mean, T_i= effect of AFB1 and e_{ij}= residual error. For gas production data, for which was repeated over time, the effects of time and time× AFB1 level were included in the REPEATED statement of the model. Significant means were compared using the Duncan's multiple range tests. Mean differences were considered significant at $P<0.05$.

3. RESULTS AND DISCUSSION

Estimated parameters of gas production are presented in Table 2 and cumulative gas production profiles are shown in Fig. 1. In this study, gas production was significantly influenced by the AFB1 level and sampling time (Fig. 1). The addition of AFB1 affected the rate and cumulative gas production ($P<0.05$), so, by increasing the level of AFB1 from 0 to 900 ng/ml, the gas production rate (c) decreased from 0.071 to 0.051 and cumulative gas production decreased from 196.4 to 166.0 ml/g DM, respectively.

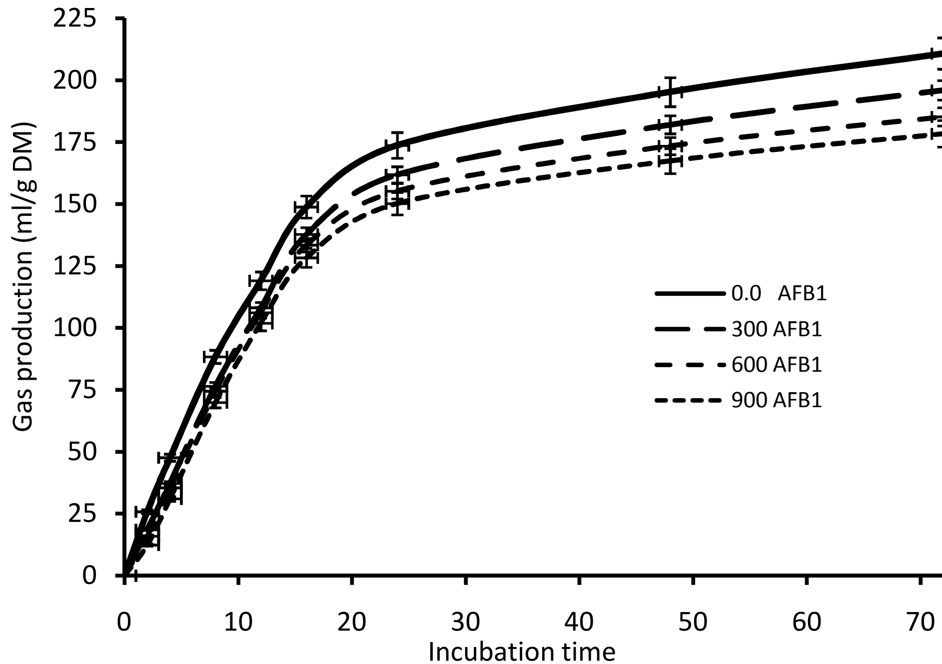

Fig. 1. Pattern of *in vitro* gas production (fitted with exponential model) affected by different levels of aflatoxin B1 (0, 300, 600 and 900 ng AFB1/ml of medium). (AFB1 effect: P<0.05; Time effect: P<0.01; AFB1×Time effect: P=0.73)

These results are consistent with those of Jiang et al. [7] and Helferich et al. [22,23], who reported that the gas production parameters were reduced when AFB1 was added. These depressions in the gas production suggest that microbial populations are altered by AFB1 contamination.

Results of present study indicated that IVDMD decreased significantly (P<0.05) with inclusion of AFB1 in culture medium, so that the lowest and the highest IVDMD values were observed in treatments with 900 and 0 ng/ml AFB1, respectively (0.54 vs. 0.68). In agreement with our result Westlake et al. [8] reported that IVDMD of alfalfa hay was reduced by 50% with inclusion of 1 µg/ml AFB1. In another study [5], cellulose digestion and ammonia formation decreased with aflatoxin levels greater than 200 ng per ml in an *in vitro* artificial rumen system. In our study decreasing IVDMD with AFB1 addition can be attributed to compromised ruminal function by reducing fiber digestion and volatile fatty acid production [5,22,23]. However some studies reported no effect of AFB1 on *in vitro* dry matter disappearance of hay [6,7].

Our results indicated that the addition of AFB1 significantly (P<0.05) decreased ammonia-N concentrations, so the lowest value was observed at 900 ng/ml AFB1, but were similar for 0 and 300 ng/ml doses (Table 2). Our result is consistent with other studies [5,7] who reported that the inclusion of AFB1 in an *in vitro* artificial rumen system decreased ammonia-N concentration. Ruminal ammonia concentration is a combined result of ammonia production, ammonia absorption and microbial ammonia uptake and utilization. The decrease in ruminal ammonia concentration with addition of AFB1 in the present study may be due to slower

release of ammonia from the diet thereby inhibition of protein digestion and metabolism by AFB1.

Table 2. *In vitro* dry matter disappearance (IVDMD), gas production (GP) parameters and ammonia-N concentrations responses to different doses of aflatoxin B1 (AFB1) of an alfalfa hay based diet using batch culture

Items	Aflatoxin B1 (ng/ml)					
	0	300	600	900	SEM	*P*-value
Gas production parameters						
GP rate (c)	0.071[a]	0.066[a]	0.054[b]	0.051[b]	0.005	0.04
Cumulative GP after 72 h (b)	196.4[a]	183.6[b]	170.5[c]	166.0[c]	6.1	<0.01
IVDMD	0.68[a]	0.64[b]	0.59[c]	0.54[d]	0.07	<0.01
Ammonia-N (mg/ 100 ml)	28.8[a]	27.6[a]	23.6[b]	18.1[c]	0.37	0.02

The nonlinear equation, GP (ml/g DM) = $b\times(1-e^{-ct})$ was used to analyze the kinetic data as described by Osuji et al. [19]; Means in the same row with different letters differ significantly; SEM: standard error of means.

Mathur et al. [24] observed that aflatoxin inhibited pure cultures of *Streptococcus bovis* and mixed rumen bacterial cultures. Burmeister and Hesseltine [25] used a crude extract of aflatoxin that contained 36 percent total aflatoxin and 24 percent aflatoxin B1 to determine the sensitivity of microorganisms to aflatoxin. Of 329 microorganisms surveyed, 12 species of *Bacillus,* a *Clostridium* spp., and a *Streptomyces* spp. were inhibited. Although crude extracts of *A. flavus* may contain several antimicrobial agents, their experiments indicated that aflatoxin B1 was the principal antimicrobial agent.

One limitation of this study is that the fermentation results observed with AFB1 may only be applicable to inordinate amounts of AFB1, which may not be naturally encountered. For example If aflatoxin is removed from the rumen at a rate similar to average rumen contents, a 600-kg cow consuming 20 kg of feed containing high AFB1 level as 900 ppb, would have approximately 200 ng aflatoxin Bl per milliliter of rumen contents.

Although our results are in agreement with some previously reports, some other studies report contradicting data. The discrepancy between these reports may be due to differences in technique, different AFB1 levels and sources of aflatoxins. For instance the procedure of Fehr and Delage (5) used 20 ml of inoculum incubated for 24 h, while Pettersson and Kiessling (6) used a procedure that incubated 1 ml of rumen fluid inoculum with the substrate for 96 h. The long incubation period and low level of initial inoculum could result in a selected microbial population that may not represent rumen conditions. In addition, long-term fermentation obscures the results of *in vitro* systems, since early differences in rate or extent of digestion would be obliterated. In other hand, there were almost certainly some other antimicrobial agents and mycotoxins, other than AFB1, as well as some nondetectable mycotoxins in the diets or crude extracts of *A. flavus* used. This may explain the variety of effects observed in studies with naturally contaminated feed compared with experiments with pure mycotoxins [26,27,28].

4. CONCLUSION

Results of present study demonstrated that the addition of different levels of AFB1 affected *in vitro* fermentation characteristic, as represented in reduced gas production, dry matter digestibility and ammonia-N concentrations. Therefore it is necessary to control and manage aflatoxin contaminations in ruminants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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