Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 20 No. 4 Ser. No. 69 2019

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

In vitro evaluation of aflatoxin B1 effect on gas production and ruminal fermentation parameters

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(Received 31 Jul 2018; revised version 31 Mar 2019; accepted 13 May 2019)

Abstract

Background: Aflatoxins are fungal secondary metabolites negatively affecting ruminant performance; however, little information is available on their impact on rumen fermentation. **Aims:** This study aimed at determining the effects of different concentrations of aflatoxin B1 (AFB1) from *Aspergillus flavus* on *in vitro* gas production and ruminal fermentation parameters using two experiments (Exp.). **Methods:** In Exp. 1, two concentration ranges (0, 0.5, 1, and 1.5 µg/ml of rumen inoculum as low and 0, 5, and 10 µg/ml as high concentration ranges) were used to evaluate AFB1 effect on gas production kinetics using 96-h incubations. In Exp. 2, only the high concentration range was used to investigate AFB1 effects on ruminal fermentation parameters using 24-h incubations. **Results:** In the low concentration range, the half-time of asymptotic gas production ($T_{1/2}$) increased and the fractional rate of gas production (µ) decreased linearly with AFB1 dosage ($P<0.05$). However, in the high concentration range, the asymptotic gas production (A) and $T_{1/2}$ decreased; and the lag time (L) and "µ" increased linearly ($P<0.001$) by increasing the concentrations of AFB1. In Exp. 2, dry matter (DM) and organic matter (OM) disappearance, microbial biomass (MB) and total volatile fatty acids (TVFA) concentrations were depressed, but pH and ammonia-N concentration increased ($P<0.01$) by increasing the concentrations of AFB1. The pattern of rumen volatile fatty acids (VFAs) was also modified by AFB1, as the propionate proportion increased at the expense of acetate. **Conclusion:** Aflatoxin B1 had an adverse effect on *in vitro* ruminal fermentation parameters in high concentration ranges (5 and 10 µg/ml).

Key words: Aflatoxin B1, *In vitro* gas production, Rumen fermentation parameters, VFA

Introduction

Aflatoxins are mainly produced by *Aspergillus flavus* and *A. parasiticus* and to a lesser extent by *A. bombycis*, *A. ochraceoroseus*, *A. nomius*, and *A. pseudotamari* (Peterson *et al*., 2001). They have the potential to infect many post-harvested crops such as cereals, forages and silages commonly used in animal feeding (Alonso *et al*., 2013; Gallo *et al*., 2015).

With the increasingly warming climate, the challenge of aflatoxins, especially that of aflatoxin B1 (AFB1) as the most potent toxin in this group, would be of higher importance since a higher crop contamination rate is observed in warmer climates (Chauhan *et al*., 2008; Jouany *et al*., 2009). Infected feeds may cause, depending on the ingested dose or the exposure time to the toxin, acute or chronic aflatoxicosis in farm animals, impairing their health and thereby reducing their performance (Jouany *et al*., 2009). Ruminants are generally more resistant to AFB1 than other farm animals such as monogastrics because of the partial degradation of AFB1 by rumen microorganisms (Hussein and Brasel, 2001; Upadhaya *et al*., 2009; Gonçalves *et al*., 2015). However, non-ruminant farm animals also possess the bacteria in their gut, and are capapble of degrading AFB1 though to a lesser extent (Bagherzadeh Kasmani *et al*., 2012). Despite being relatively resistant at low doses, ruminants are also vulnerable to AFB1 at relatively high doses; nevertheless, data on AFB1 toxicity in ruminants are inconsistent. In this context, while some researchers reported no toxicity from AFB1 at concentrations of up to 300 µg/kg diet (Queiroz *et al*., 2012; Xiong *et al*., 2015), others have found AFB1 to be toxic for ruminants at doses even lower than 108.5 µg/kg diet (Choudhary *et al*., 1998; Charoenpornsook and Kavisarasai, 2006). Depressed performance and feed efficiency of ruminants in these studies have been attributed to the negative impact of AFB1 on rumen microbial ecosystems. However, there is limited information about the direct impact of AFB1 on rumen microbial activities. Moreover, the data in the literature is controversial. While, Mojtahedi *et al*. (2013) reported a negative impact of AFB1 on rumen functions at rumen fluid concentrations of 300-900 ng/ml, no adverse effect of AFB1 on ruminal digestibility and gas production was observed by Jiang *et al*. (2012) at similar concentrations. At doses that typically induce chronic aflatoxicosis,

AFB1 reduces feed intake and affects the performance of ruminants without affecting rumen volatile fatty acid (VFA) concentrations (Helferich *et al*., 1986a; Edrington *et al*., 1994), suggesting that AFB1 toxic doses are probably higher for rumen microbial ecosystems than those for animal health or performance. Thus, determining AFB1 toxic concentrations for rumen microbial ecosystems and elucidating its effect on rumen fermentation can give a clear picture of its overall impact on ruminant health and productivity.

The objective of the present study was to determine the effect of different dosages of AFB1, from *A. flavus*, on *in vitro* gas production and ruminal fermentation parameters.

Materials and Methods

Experiments (Exp.)

This research consisted of two Exp.; in Exp. 1 the objective was to assess the impact of AFB1 at different concentrations on *in vitro* gas production kinetics as an indicator of rumen microbial activity using two sets of 96-h incubations. For this, a wide range of AFB1 concentrations was tested: 0 as control (CTRL), 0.5, 1 and 1.5 µg/ml (as low concentration) in the first set of incubations, and 0, 5, and 10 µg/ml (as high concentration) in the second set of 96-h incubations. In Exp. 2, due to its significant effect on gas production kinetics, only the high concentration range of AFB1 was selected to investigate its effects on ruminal dry matter (DM) and organic matter (OM) disappearance and fermentation using 24-h incubations.

Aflatoxin solutions

The AFB1 pure extract from *A. flavus* (10 mg powder, purity >99%) was procured from Sigma-Aldrich (St. Louis, MO, USA), dissolved in 5 ml absolute ethanol (Merck, Darmstadt, Germany) (Upadhaya *et al*., 2010) and subsequently diluted at the ratios of 1:25.67, 1:12.33, 1:7.89, 1:1.67, and 1:0.33 (for final doses of 0.5, 1, 1.5, 5, and 10 µg/ml) with sterilized deionized water and ethanol (the proportion of ethanol remained the same in all the solutions) to provide the working solutions with appropriate concentrations of AFB1 used in the Exp. (Upadhaya *et al*., 2010).

Animals and rumen fluid

Rumen contents were collected before the morning feeding from three ruminally fistulated mature Mehraban rams (50 ± 4 kg body weigh (BW)). The rams were fed *ad libitum* with a total mixed ration of (DM basis) 50% alfalfa hay; 7% wheat straw; 37% barley; 4% cotton seed meal; 1% NaCl, and 1% mineral and vitamin supplements. Estimated metabolizable energy and crude protein (CP) content of the diet were 9.5 mega joule (MJ) and 12.2%, per kg DM respectively (NRC, 1985). Rumen contents were then pooled, strained through four layers of cheesecloths into a pre-warmed insulated flask and immediately transported to the laboratory.

In vitro gas production

The *in vitro* gas production procedure was conducted as described by Menke and Steingass (1988). Incubated substrates were the same as those fed to the rams. In 96-h incubations, a representative air-dried sample was ground to pass a 1 mm sieve and sub-samples of 200 mg (DM basis) were weighed into 100 ml glass syringes. Incubation of the samples was conducted in triplicate with 30 ml of buffered rumen inoculum (prepared by mixing rumen fluid with the buffer at a ratio of 1:2 (v/v) under the continuous flow of CO_2). Three syringes containing 30 ml of the buffered rumen fluid without substrate were considered as blanks. After adding AFB1 (200 µL of each AFB1 working solution to obtain final concentrations of 0, 0.5, 1, and 1.5 µg/ml in the first and 0, 5, and 10 µg/ml of the cultures in the second set of incubations), the syringes were placed in a water-bath at 39°C and the gas volume was recorded at 2, 4, 6, 8, 12, 24, 48, 72, and 96-h of incubation. In Exp. 2, a larger amount (500 mg) of the substrate was incubated in 100 ml glass syringes with AFB1 (final concentrations of 0, 5, and 10 µg/ml) containing 40 ml of buffered rumen fluid, as described by Makkar *et al*. (1995). Incubation with a larger amount of the substrate was done in order to avoid inherent gravimetric errors commonly associated with a small sample size (Blümmel and Becker, 1997). After 24-h of incubation, the content of the syringes was transferred into centrifuge tubes and immediately placed in cold water at 4°C to stop the fermentation. The tube content was then centrifuged at 15000 × g for 20 min at 4°C, aliquots of 4 ml from the supernatant were mixed with 1 ml of 25% metaphosphoric acid and frozen at -20°C until subsequent analysis for VFA and ammonia content (Nocek *et al*., 1987; Kahvand and Malecky, 2018). Remaining residues in the tubes were oven-dried at 60°C for 48-h to estimate *in vitro* apparent dry matter degradability (IVADMD). *In vitro* true organic matter degradability (IVTOMD) was determined by refluxing the oven-dried residue with neutral detergent solution at 100°C for 1-h, with subsequent incinerating of the recovered substrate in sintered glass crucibles at 600°C (Makkar *et al*., 1995).

Chemical analyses

Standard methods, as described in AOAC (2000), were used to determine DM, total ash, ether extract (EE) and CP. Neutral detergent fibre (NDF) was determined according to Van Soest *et al*. (1991). Ammonia concentration in the supernatants was determined as illustrated by Broderick and Kang (1980). Volatile fatty acids (VFAs) concentrations of the samples were quantified according to Ottenstein and Bartley's (1971) method, using a gas chromatograph (GC-FID, PU4410-PHILIPS, England) equipped with a flame ionization detector and a 10PEG column (1.8 m × 4.6 mm i.d., glass column packed with 10% SP 1,200, 1% H_3PO_4 on 80/100 chromosorb WAW). Nitrogen was used as the carrier gas at a constant flow rate of 35 ml/min. The oven temperature was programmed as follows: initial

temperature of 100°C, held for 4 min; increased at 5°C min-1 to 135°C, and then at 10°C/min to 200°C and held at 200°C for 20 min. The injector and detector temperature was set at 210°C.

Calculations and statistical analysis

Data on the cumulative gas production during 96-h of incubation were fitted to the model proposed by France *et al*. (1993) as shown below, by none linear (NLIN) procedure of SAS (2002).

$$GP = A\left\{1 - e^{-\left[b(t-L)+c\left(\sqrt{t}-\sqrt{L}\right)\right]}\right\}$$

Where,
GP (ml): The gas produced at the time t
A (ml): The asymptotic gas production
b and c: Constant coefficients
L (h): The lag time

The half-times of asymptotic gas production ($T_{1/2}$, h) and fractional gas production rate at $T_{1/2}$ (μ, h^{-1}) were calculated using the equations proposed by France *et al*. (1993). The ratio of the organic matter truly degraded (mg) to the gas produced (ml) after 24-h of incubation was used as the partitioning factor (PF) (Blümmel and Lebzien, 2001). The mass difference between the oven-dried residue and that recovered after neutral detergent extraction was calculated and considered as MB.

All the Exp. were repeated twice during two different days (run). Data in each Exp. were subjected to the analysis of variance by the general linear model (GLM) procedure of SAS (2002) using a combined analysis. The model included treatment (fixed effect), run (day), treatment × run, and replications nested in day as random effects (in total 6 replications per treatment level). A specific orthogonal contrast used to evaluate the averaged effect of AFB1 against the CTRL and the trend of AFB1 concentration effect on the variables were tested by linear and quadratic contrasts.

Results

Effect of AFB1 on gas production kinetics

In the low concentration range (0, 0.5, 1, and 1.5 μg/ml), additing AFB1to the cultures had no effect on asymptotic gas production and "L" (P>0.05) (Table 1). However, $T_{1/2}$ increased and "μ" decreased linearly by increasing the concentration of AFB1 (P<0.05).

In the high concentration range (0, 5, and 10 μg/ml), all kinetic parameters were affected by AFB1 (Table 2). The asymptotic gas production decreased in both linear and quadratic manners by increasing the concentration of AFB1 (P<0.05). However, the "L" and "μ" increased nonlinearly when the AFB1 concentration increased in the cultures (P<0.05). The $T_{1/2}$ increased linearly by increasing the concentration of AFB1 (P<0.001).

Effect of AFB1 on *in vitro* ruminal substrate disappearance and fermentation

The gas produced after 24-h of incubation decreased in both linear and quadratic manners (P<0.001), and the lowest value was observed with the highest concentration of AFB1 (Table 3). The IVADMD and IVTOMD were negatively affected (P<0.001) by the inclusion of AFB1 in the cultures, as both were depressed in linear and quadratic manners (P<0.05) with the AFB1 dosage. However, PF increased linearly and quadratically (P<0.01) by increasing the concentration of AFB1. Despite enhancing PF, AFB1 dosage had negative linear and quadratic effects on MB (P<0.001).

Effect of AFB1 on pH, ammonia-N and the VFA pattern

The *in vitro* ruminal pH increased linearly (P<0.01) by increasing the concentration of AFB1 (Table 4). The same effect was observed on the *in vitro* ruminal

Table 1: Effects of AFB1 in the low concentration range on gas production kinetics

Parameters[1]	AFB1 concentration (μg/ml)				SEM	P-values (contrasts[2])		
	0	0.5	1	1.5		CTRL vs. AFB1	L	Q
A (ml)	69.9	68.8	76.5	78.4	3.87	0.358	0.124	0.718
L (h)	0.51	0.63	0.37	0.31	0.078	0.494	0.070	0.302
$T_{1/2}$ (h)	5.7	5.1	7.1	7.5	0.52	0.221	0.027	0.358
μ (/h)	0.09	0.11	0.07	0.06	0.010	0.420	0.035	0.205

[1] Parameters of gas production kinetic were estimated using the model proposed by France *et al*. (1993), A: Asymptotic gas production (ml per 200 mg DM), L: Lag time, $T_{1/2}$: Half-time of asymptotic gas production, μ: Fractional rate of gas production, AFB1: Aflatoxin B1, SEM: Standard error of the means, [2] CTRL: Control, 0 μg/ml of AFB1, L: Linear, and Q: Quadratic

Table 2: Effects of AFB1 in the high concentration range on gas production kinetics

Parameters[1]	AFB1 concentration (μg/ml)			SEM	P-values (contrasts[2])		
	0	5	10		CTRL vs. AFB1	L	Q
A (ml)	70.3	52.5	42.1	0.88	<0.001	<0.001	0.02
L (h)	0.53	0.97	1.05	0.031	<0.001	<0.001	0.006
$T_{1/2}$ (h)	5.5	4.7	3.4	0.10	<0.001	<0.001	0.08
μ (h^{-1})	0.09	0.13	0.22	0.007	<0.001	<0.001	0.038

[1] Parameters of gas production kinetic were estimated using the model proposed by France *et al*. (1993), A: Asymptotic gas production (ml per 200 mg DM), L: Lag time, $T_{1/2}$: Half-time of asymptotic gas production, μ: Fractional rate of gas production, AFB1: Aflatoxin B1, SEM: Standard error of the means, [2] CTRL: Control, 0 μg/ml of AFB1, L: Linear, and Q: Quadratic

Table 3: Effects of AFB1 on ruminal 24-h gas production and degradability

Variables[1]	AFB1 concentration (µg/ml)			SEM	P-values (contrast[2])		
	0	5	10		CTRL vs. AFB1	L	Q
GP_{24} (ml)	125.1	79.7	67.9	1.18	<0.001	<0.001	<0.001
IVADMD (%)	48.2	43.5	41.9	0.36	<0.001	<0.001	0.014
IVTOMD (%)	62.9	55.5	54.4	0.45	<0.001	<0.001	0.002
PF	2.3	3.2	3.7	0.03	<0.001	<0.001	0.004
MB (mg)	73.4	60.0	62.3	0.54	<0.001	<0.001	<0.001

[1] GP_{24}: Gas produced after 24-h of incubation (ml per 500 mg DM), IVADMD: *In vitro* apparent dry matter degradability, IVTOMD: *In vitro* true organic matter degradability, PF: Partitioning factor, MB: Microbial biomass, AFB1: Aflatoxin B1, SEM: Standard error of the means, [2] CTRL: Control, 0 µg/ml of AFB1, L: Linear, and Q: Quadratic

Table 4: Effects of AFB1 on pH, ammonia-N concentration and VFAs molar proportions after 24-h of incubation

Variables[1]	AFB1 concentration (µg/ml)			SEM	P-values (contrasts[2])		
	0	5	10		CTRL vs. AFB1	L	Q
pH	6.32	6.41	6.44	0.015	0.002	0.003	0.169
$NH_{3\text{-}N}$ (mmol/ml)	15.7	16.6	17.5	0.22	0.003	0.003	0.876
TVFA (mmol/ml)	66.8	52.8	45.4	2.10	<0.001	0.001	0.242
VFA molar proportion (mmol/100 mmol)							
Acetate (A)	66.3	54.3	53.5	0.90	<0.001	<0.001	0.003
Propionate (P)	19.7	27.7	25.8	0.40	<0.001	<0.001	<0.001
Butyrate	9.7	10.8	13.6	0.76	0.388	0.017	0.368
Isovalerate	1.93	4.29	3.30	0.403	0.011	0.069	0.017
Valerate	1.70	2.95	2.94	0.278	0.013	0.029	0.112
P/A	0.30	0.51	0.49	0.007	0.007	<0.001	<0.001

[1] TVFA: Total volatile fatty acids, P/A: Propionate to acetate ratio, VFAs: Volatile fatty acids, AFB1: Aflatoxin B1, SEM: Standard error of the means, [2] CTRL: Control, 0 µg/ml of AFB1, L: Linear, and Q: Quadratic

ammonia-N concentration. However, the total volatile fatty acids (TVFA) concentration decreased linearly (P<0.01) with AFB1 dosage.

Molar proportions of VFA were also altered by AFB1; acetate molar proportion decreased linearly and quadratically (P<0.01), as the lowest value was detected with the highest concentration of AFB1. Conversely, propionate molar proportion increased with linear and quadratic trends (P<0.001) with AFB1 dosage, with the highest value observed at the lowest concentration of AFB1. The molar proportion of butyrate did not differ among the treatments, but those of valerate and isovalerate increased by increasing the concentration of AFB1. The propionate to acetate ratio increased by increasing the concentration of AFB1 (P<0.001) in linear (P<0.001) and quadratic (P<0.001) manners, with the highest value recorded at low concentrations of AFB1.

Discussion

Effect of AFB1 on gas production kinetics

Results of Exp. 1 revealed that AFB1 had no significant impact on asymptotic gas production, indicating that the *in vitro* ruminal fermentation extent remained unaffected by AFB1 at concentrations of up to 1.5 µg/ml. These results are in line with those obtained by Jiang *et al*. (2012), who observed no change in asymptotic gas production when AFB1 was added at concentrations of 320 to 960 ng/ml. In contrast, Mojtahedi *et al*. (2013) reported a decreased asymptotic gas production with AFB1 at concentrations of 300 to 900 ng/ml. Previous studies have also shown an inconsistency in the impact of AFB1 on farm animal health and productivity. In this context, while in some Exp. AFB1 had no adverse effect on steers and lambs at doses up to 600 and 2500 µg/kg diet, respectively (Helferich *et al*., 1986a; Edrington *et al*., 1994), in others Exp., AFB1 showed to be toxic at doses around 100 µg/kg diet. These contradictions may be due to the difference in the rumen fluid incubated, the diet fed to the animals, the aflatoxin source, and different conditions of the Exp. (Mojtahedi *et al*., 2013; Gallo *et al*., 2015).

In the current study, the decreased "µ", as well as the longer $T_{1/2}$ resulting from the increased concentration of AFB1 from 0 to 1.5 µg/ml, could be indicative of a lower fermentation rate caused by a decreased rumen microbial activity in the presence of AFB1. Similarly, Jiang *et al*. (2012) and Mojtahedi *et al*. (2013) reported a decreased "µ" at concentrations of 0-900 ng/ml of AFB1.

In the high concentration range of AFB1 in Exp. 2, the decreased asymptotic gas production caused by the AFB1 dosage demonstrated that AFB1 had a lowered fermentation extent. However, the higher "µ" accompanied with the shorter $T_{1/2,}$ could be a result of the selective inhibitory effect of AFB1 on some rumen microorganisms, mainly cellulolytic bacteria characterized with a low growth rate and fermentation activity (Dehority, 2003). Indeed, "µ" is an index of the fermentation rate proportional to the substrate degradation rate, which is determined by fermentation substrate type and rumen dominant microorganisms

(France *et al*., 2000). Hence, a higher "μ" was expected in a culture dominated eventually by rapid fermenting bacteria caused by the selective effects of AFB1 on rumen microbial ecosystems.

The results of Exp.1 revealed that AFB1 has no considerable impact on rumen fermentation at concentrations up to 1.5 μg/ml as the gas production kinetic parameters did not change compared with the CTRL. However, at 5 and 10 μg/ml of AFB1 dosage, a lower asymptotic gas production accompanied by a high gas production rate implies a selective negative impact of AFB1 on rumen fermentation.

Effect of AFB1 on *in vitro* substrate disappearance and fermentation parameters

The significant decrease in IVADMD and IVTOMD with AFB1 dosage is most probably the principal cause of the great drop in GP_{24}. The result is consistent with those obtained by Westlake *et al*. (1989) who reported a decrease in *in vitro* substrate degradability with AFB1 at 1 and 10 μ/ml. Similarly, Jiang *et al*. (2012) and Mojtahedi *et al*. (2013) reported a decreased *in vitro* ruminal digestibility with AFB1 at concentrations of 300-960 ng/ml. A similar negative effect of other mycotoxins on ruminal digestibility has been reported previously (Morgavi *et al*., 2003). In the current study, the decrease in GP_{24} was more pronounced than that of IVTOMD, resulting in a high PF with 5 and 10 μ/ml of AFB1. This might partially be due to a part of OM dissolved in the culture that was not fermented by the rumen microorganisms (Getachew *et al*., 2000; Makkar, 2003). Moreover, it has been shown that a higher PF value is related to a higher propionate to acetate ratio, because direct gas produced from OM fermentation comes only from an acetate producing pathway (Getachew *et al*., 1998). The VFA pattern in our study was also in line with these findings. Therefore, a higher PF could be anticipated as a result of a higher propionate to acetate ratio (Blümmel *et al*., 1999). A lower MB with AFB1 dosage reveals that the growth of some rumen microbial populations was inhibited (Fink-Gremmels, 2008; Jiang *et al*., 2012). This might be a result of the direct impact of AFB1 on some rumen microorganisms through which it impairs their protein synthesis (Edrington *et al*., 1994).

Effect of AFB1 on *in vitro* ruminal pH, ammonia-N and VFA concentrations

The linear increase in the *in vitro* ruminal pH with AFB1 concentration increases is probably related to the increased ammonia and decreased TVFA concentration (Clark and Lombard, 1951; Coombe and Tribe, 1962). The decreased TVFA caused by including AFB1 in the cultures was consistent with the drop in IVTOMD and GP_{24} indicating a partial inhibition of rumen microorganisms (Blümmel *et al*., 1997; Jiang *et al*., 2012). Higher molar proportions of propionate, as well as the higher propionate to acetate ratio and the inclusion of AFB1 in the culture (especially at a low concentration (5 μg/m)), could be indicative of a shift in rumen bacterial compositions in favor of propionate producing bacteria. In an earlier study, Morand Fehr and Delag (1970) reported a similar rumen VFA pattern (lower acetate and higher propionate proportion), but with lower concentrations of AFB1 (0.36 and 0.6 μg/ml).

Valerate, isovalerate and ammonia are end-products of protein fermentation (Zhang *et al*., 2013). Additionally, they are considered as important growth factors required mainly for cellulolytic bacteria (Atasoglu *et al*., 2001; Zhang *et al*., 2013). Therefore, ruminal concentrations of these end-products at any given time is a balance between their production by rumen proteolytic microorganisms and their uptake by cellulolytic bacteria (Nolan and Dobos, 2005). Hence, the higher concentration of valerate, isovalerate and ammonia observed in AFB1-contained cultures might be a consequence of the decreased growth or inhibition of cellulolytic bacteria (caused by AFB1) rather than higher rumen proteolytic activity, especially considering depressed OM and DM degradabilities in these cultures. On the other hand, regarding the fact that microbial recycling in the rumen contributes to ruminal ammonia concentrations, high ammonia concentrations might also be a result of a higher microbial lysis caused by AFB1 (Wells and Russell, 1996; Hackmann and Firkins, 2015).

The results of the present study showed that AFB1 had no marked adverse effect on *in vitro* rumen fermentation parameters at concentrations up to 1.5 μg/ml. However, in high concentration ranges (5 and 10 μg/ml), *in vitro* gas production, and rumen fermentation were adversely affected by AFB1.

Acknowledgements

This research was supported by a thesis grant for M. Khodabandehloo from Bu-Ali Sina University; the authors would like also to thank Dr. Zamani for his statistical advice.

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