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Comparative study on the aflatoxin B1 degradation ability of rumen fluid from Holstein steers and Korean native goats

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The aflatoxin B1 degrading abilities of two different ruminants were compared in this study. One set of experiments evaluated the aflatoxin B1 degradation ability of different rumen fluid donors (steers vs. goats) as well as the rumen fluid filtration method (cheese cloth filtered vs. 0.45 μm Millipore) in a 2 × 2 factorial arrangement. Additional studies examined aflatoxin B1 degradation by collecting rumen fluid at different times (0, 3, 6, 9 and 12 h) after feeding. Cannulated Holstein steers (740 ± 10 kg bw) and Korean native goats (26 ± 3 kg bw) were fed a 60% timothy and 40% commercial diet with free access to water. Rumen fluid from Korean native goats demonstrated higher ($p < 0.01$) aflatoxin B1 degradability than Holstein steers. However, filtration method had no significant influence on degradability. In addition, aflatoxin degradation did not depend upon rumen fluid collection time after feeding, as no significant differences were observed. Finally, a comparison of two types of diet high in roughage found aflatoxin degradability in goats was higher with timothy hay opposed to rice straw, although individual variation existed. Thus, our findings showed the aflatoxin degradability is comparatively higher in goats compared to steers.

Keywords: aflatoxin B1, degradation, ELISA, rumen

Introduction

Aflatoxin is one of several extremely toxic, mutagenic and carcinogenic compounds produced by *Aspergillus* (*A.*) *flavus* and *A. parasiticus* [6]. Research studies have revealed four major aflatoxins; B1, B2, G1 and G2 as well as two additional metabolic products, M1 and M2, that are direct contaminants of foods and livestock feed. Of these, aflatoxin B1 (AFB1) is the most prevalent and toxic for both animals and humans [17].

Aflatoxin interferes with disease resistance and vaccine-induced immunity in livestock [7], exemplified by immunity suppression by AFB1 observed in turkeys, chickens, pigs, mice, guinea pigs, and rabbits [22]. Symptoms of acute aflatoxicosis in mammals include inappetence, lethargy, ataxia, rough hair coat, and enlarged pale fatty livers. In contrast, chronic aflatoxicosis exhibits symptoms including reduced feed efficiency and milk production, icterus, and decreased appetite [18]. Reduced growth rate is possibly the most obvious indication for chronic aflatoxicosis and other mycotoxicoses [20] and is related to disturbances in protein, carbohydrate and lipid metabolism [3].

Aflatoxins have been detected in numerous agricultural commodities such as cereal grains, oilseeds, cotton seeds, wheat, corn, peanuts and dried fruits as well as in animal feed and various dairy products [19]. The toxin becomes stable once formed in grain, resistant to degradation during normal milling and storage [2]. This presents the toxicity of contaminated feed stuffs as a significant, potential health hazard to animals and human beings.

Several strategies for the decontamination/detoxification of grains contaminated by mycotoxins have been reported using physical, chemical and biological methods specific to the commodity. However, previous treatments have exhibited limitations due to considerations for safety, which require not only the treated products be unaffected by the chemicals used, but also that their essential nutritive values be maintained [16]. A study by Wang *et al*. [23] found adsorbents like activated charcoal and hydrated sodium aluminum silicates at low percentage were ineffective when used to treat moldy feed. It was observed a high percentage of adsorbents bind essential nutrients, causing negative effects.

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The application of enzymes or microorganisms capable of biotransforming mycotoxins into nontoxic metabolites has emerged as an alternative strategy in controlling mycotoxicoses in animals. Microbes transform mycotoxins in the intestinal tract of animals prior to absorption. Biotransformation, the cleavage and detoxification of mycotoxin molecules by microbes or enzymes, is an effective and safer method for mycotoxin control [21].

Several mycotoxins and plant toxins have been shown previously to be detoxified by rumen microbes, ochratoxin A (OTA) [9] and AFB1 [1] among the first. Jones *et al*. [10] reported the disappearance of AFB1 within several weeks of incubation with broiler and turkey faeces. Karlovsky [13] reported a 42% degradation of aflatoxin when incubated *in vitro* with rumen fluid. The ability of ruminants to metabolize selected mycotoxins has also been investigated [14]. It was found the mycotoxins zearalenone (ZON), trichothecenes mycotoxin (T-2 toxin), diacetoxyscirpenol and deoxynivalenol were well-metabolized by whole rumen fluid, whereas AFB1 and OTA were not. Westlake *et al*. [24] investigated the effects of these mycotoxins along with Verrucarin A on the growth rate of *Butyrivibrio* (*B.*) fibrisolvens specifically. They found this organism degraded all tested mycotoxins except AFB1, and that growth of *B. fibrisolvens* was not inhibited. Kurmanov [15] previously reported ruminants are more resistant to mycotoxin poisoning than monogastrics, which implies livestock are not equally affected by other toxins as well. *In vitro* rumen fermentation studies on the plant toxin pyrrolizidine alkaloid (PA) showed a higher degradation ability in sheep and goat than cattle [4,8]. Likewise, animals fed tansy ragwort containing PA demonstrated vastly different quantities of plant material required to manifest clinical symptoms. The consumption at the rate of more than 200% of the body weight of sheep and goat, 4-10% of the body weight of cattle and horse and 5% of the body weight of chicken was required to show clinical signs [5,8].

In this study, aflatoxin degradation in Holstein steers and Korean native goats was examined using rumen fluid as a microbial source. Our intent is to use the findings for the future selection of potential ruminant species containing bacteria having aflatoxin degradation ability.

Materials and Methods

Experimental animal and diet

Three cannulated Holstein steers (740 ± 10 kg body weight) and three Korean native goats (26 ± 3 kg body weight) served as rumen fluid donors. Animals were maintained on 40% concentrates comprised of 16.5% crude protein (Corn beef; Purina, Korea) and 60% roughage (timothy hay; Feed land, USA).

To assess the effect of different substrates (rice straw and timothy) on aflatoxin degradability after incubation at 39°C, roughage-based diets (80 : 20) were fed to three goats followed by rumen fluid collection and supplementation with pure AFB1 extract.

Aflatoxin

Pure extract of AFB1 (10 mg powder) was procured from Sigma-Aldrich (USA) and dissolved in absolute ethanol (Merck KGaA, Germany). Dilutions were performed in sterilized distilled water for preparation of the working solution, the concentration of which was further diluted in order to be within the detection range of the kit (AgraQuant Total Aflatoxin Test kit (4-40 ppb) (Romer Labs, Singapore). The concentration in parts per billion (ppb) was determined using an ELISA reader (Biotrak II; Amersham Biosciences, UK) at 450 nm wavelength filter.

Rumen fluid collection

Rumen contents were collected through a canula 1 h after morning feeding in a 500 ml stainless steel vacuum bottle and immediately transferred to laboratory. Rumen fluid containing the ingesta was subjected to oxygen-free CO_2 using a gassing apparatus, homogenized with a mixer (Mini mixer; Hanil, Korea) for 1 min, then strained through 8-layer cheese cloth for further experimentation. To investigate aflatoxin degradation based on sampling time after feeding, rumen fluid was collected in 15 ml sterilized falcon tubes in triplicates and immediately innoculated into sterilized Hungate tubes containing aflatoxin. Incubation was done in different time points.

Experimental design

Experiment A: AFB1 degradation ability of rumen microorganisms from cattle and goat

The purpose of this study was to investigate variation in AFB1 degradability among different species along with the effect of different types of rumen fluid on toxin degradation. We employed a 2 × 2 factorial arrangement consisting of the rumen fluid donors (steers *vs.* goats) versus the rumen fluid preparation method (cheese cloth filtered vs. 0.45 μm Millipore [Advantec MFS, Japan] filtered rumen fluid). Rumen fluid from donor animals was strained through the eight-layer cheese cloth into sterilized Hungate tubes, giving a total sample volume with aflatoxin of 5 ml and a final AFB1 concentration of 80 ppb. Degradation of AFB1 was measured after 3 h of incubation at 39°C without agitation, performed in triplicate. One part was used for treatment, one was cheese-cloth filtered, and the other part was centrifuged at 160 × g (Supra K21, High Speed centrifuge; Hanil Science Industrial, Korea) for 5 min, followed by filtration of supernatant with Millipore filter size (0.45 μm). Rumen fluid was autoclaved, supplemented with aflatoxin and incubated under the same

conditions for a control.

Experiment B: Effect of rumen fluid collection time on aflatoxin degradation

The effect on AFB1 degradation of rumen fluid collected at different times after feeding was investigated. Briefly, rumen fluid from donors was added to sterilized Hungate tubes to a total volume of 5 ml, supplemented with aflatoxin to a final concentration of 100 ppb then sealed with screw caps fitted with butyl rubber stoppers (Bellco Glass, USA). Aflatoxin degradation was assayed in triplicate using rumen fluid from three steers and three goats collected at different times (0 h, 3 h, 6 h, 9 h and 12 h) after feeding. Aflatoxin-containing tubes were inoculated with rumen fluid samples for each time period followed by incubation for 12 h in a shaker with the speed of 120 rpm at 39°C.

Experiment C: Effect of feed type on aflatoxin degradation

To examine the effect of feed type on aflatoxin degradation, whole rumen fluid from three goats was assayed for aflatoxin degradation like above, except under different feeding conditions. In this experiment, goats were fed 80% roughage, either timothy hay or rice straw, and 20% concentrates. Rumen fluid from donors was filled to a final volume of 5 ml in sterilized Hungate tubes, as before, then supplemented with AFB1 to a final concentration of 100 ppb (Bellco Glass, USA). The aflatoxin degradation assay was performed with rumen fluid collected at different times (3, 6, 9 and 12 h) after feeding. All tubes were incubated for 12 h in a shaker at 120 rpm and 39°C.

Sample preparation and extraction for aflatoxin analysis

The aflatoxin spiked rumen fluid sample from each incubation tubes were centrifuged at first. Then for AFB1 extraction, 300 µl supernant were taken in eppendorf tubes and mixed thoroughly with 700 µl of 100% HPLC grade methanol (Sigma-Aldrich, Germany) by vortexing. Samples that were not analyzed were immediately stored at -20°C until analysis.

Extracted AFB1 samples were diluted with 70% methanol and aflatoxin assay was done using the AgraQuant Total Aflatoxin Test Kit (4-40 ppb) (Romer Labs, Singapore).

Sample assay procedure

One hundred µl of aflatoxin test kit standards (0 ppb, 4 ppb, 10 ppb, 20 ppb and 40 ppb) were mixed with 200 µl of conjugate in individual dilution well. Similarly 100 µl of samples to be analyzed were mixed with 200 µl of conjugate in individual dilution wells. Next 100 µl from each dilution well was transferred to a respective antibody-coated microwell. Incubation for 15 min at room temperature was followed by washing each well 5 times with distilled water then tap-drying with several layers of absorbent paper. Enzyme substrate (100 µl) was added to each well and incubated for an additional 5 min. Stop solution (100 µl for each well) was added lastly and the intensity of the resulting yellow color was measured optically with a microplate reader at a wavelength of 450 nm. The total incubation time of the test kit assay was 20 min.

Absorbances obtained from the plate reader were interpolated to the Romers Labs (Singapore) data reduction spread sheet for the calculation of AFB1 concentration for each sample. The obtained ppb was multiplied by 2/3 because we added 300 µl liquid sample + 700 µl 100% methanol during aflatoxin extraction from the rumen fluid, giving a dilution factor of 10/3. The standards were prediluted by a factor of 5 (for aflatoxin kit), as indicated in the protocol of Romer Labs (Singapore). Therefore, in order to obtain a final ppb figure, we took the total dilution factor into consideration = (10/3) × (1/5) = 2/3.

Statistical analysis

All data generated were analyzed by ANOVA procedure of SAS, 2002 (SAS, USA). Differences among means were tested using the least significant difference procedure. Significance was declared at $p < 0.05$.

Results

AFB1 degradation by rumen microorganisms from cattle and goat

AFB1 degradation was observed in both species of ruminants after 3 h of incubation (Table 1). Rumen fluid obtained from Korean native goats demonstrated higher ($p < 0.01$) AFB1 degradation than that from Holstein steers. Importantly, there were no statistically significant differences between filtering methods although numerical differences were observed.

Effect of rumen fluid collection time on aflatoxin degradability

Rumen fluid supplemented with aflatoxin had higher (p

Table 1. Effect of rumen fluid filtration method and rumen fluid source on aflatoxin B1 (AFB1) degradation

Steer		Goat		SE	*p* value		
CCF	MPF	CCF	MPF		A	FM	A/FM
8.51	4.55	27.54	10.49	3.27	0.01*	0.169	0.56

AFB1 degradation (%) assay was performed by ELISA. The final concentration of aflatoxin in rumen fluid was 80 ppb. CCF: cheese cloth filtered, MPF: millipore filtered, A: animal, FM: filtering method, A/FM: interaction of animal (rumen fluid donor) and filtering method, SE: standard error, *significantly different.

< 0.01) degradability when derived from goats than from Holstein steers.

Degradation of aflatoxin in rumen fluid from goats and steers was assessed after feeding. Degradation in goats seemed to decrease immediately after feeding. However, with the increase in time after feeding, aflatoxin degradation gradually increased and reached maximum at 9 h of feeding (Fig. 1). In contrast, aflatoxin degradation in steers steadily increased from 0h to 12 h after feeding. The degradation reached maximum at 12 h after feeding. Similar to previous experiments, aflatoxin degradation was about 20% for goats and 14% for steers.

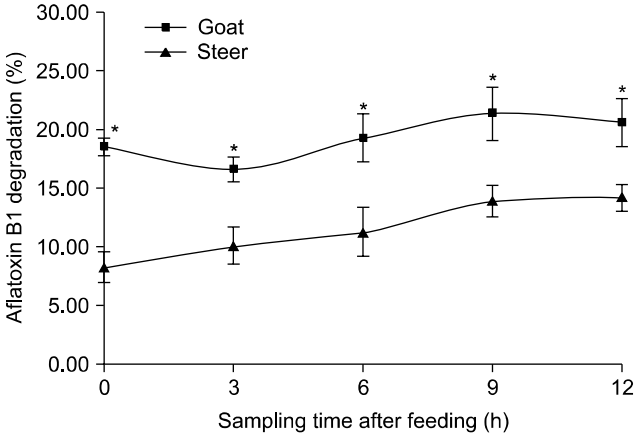

Fig. 1. Effect of rumen fluid collecting time (0, 3, 6, 9 and 12 h) after feeding on aflatoxin B1 (AFB1) degradation. Rumen fluid was supplemented with AFB1 to a final concentration of 100 ppb. *Significantly different between goat and steer ($p < 0.01$). Incubation of rumen fluid was done at 39°C and collected at times of 0, 3, 6, 9, and 12 h after feeding the animal. AFB1 degradation assay was performed by ELISA.

Effect of feed type on aflatoxin degradation

In our study, there existed individual variation with regards to AFB1 degradation within the same species. We observed that overall aflatoxin degradability tended to be higher at sampling time of 12 h of feeding in microbial source from goats fed timothy than rice straw. However individual variation did exist among the goats for degradation in different sampling times. In the microbial source from goats fed timothy hay at 12 h of feeding, goat 1 and 3 had significantly higher ($p < 0.05$) degradation than goat 2 (Fig. 2B). In rice straw fed goats, there was not much individual difference in toxin degrading ability after 12 h of feeding (Fig. 2A).

Discussion

Goats demonstrated higher (about 20-25%) AFB1 degradability than steers (10-14%) when the donor animals were fed a roughage:concentrate mixture (60 : 40). Kiessling *et al*. [14] has previously suggested mycotoxins are not completely degraded and furthermore, the extent of degradation tends to vary between different species, age, sex and breed. This could be attributable to the types of microbes inhabiting the rumen. Some studies have observed goats native to places where *Leucaena* grew were able to eat the plant, whereas domestic animals introduced to the same areas became ill and in some cases died [11].

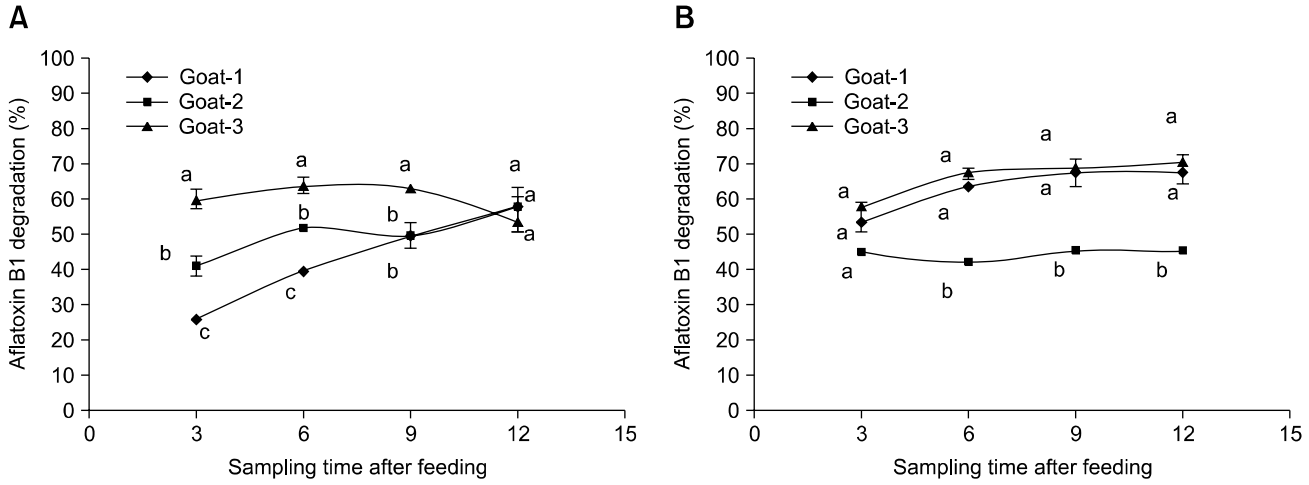

Fig. 2. (A) Effect of rice straw diet on aflatoxin degradation (%) in three goats. Three goats were fed roughage-based diets (rice straw). Rumen fluid collected at 3, 6, 9 and 12 h after feeding was supplemented with aflatoxin B1 (AFB1) to a final conc of 100 ppb. Incubation was done for 12 h at 39°C. Aflatoxin degradation assay was performed by ELISA. Means with different superscripts $^{(a,b,c)}$differ significantly ($p < 0.05$). (B) Effect of timothy hay diet on aflatoxin degradation (%) in three goats. Three goats were fed roughage-based diets (timothy hay). Rumen fluid collected at 3,6, 9 and 12 h after feeding was supplemented with AFB1 to a final concentration of 100 ppb. Incubation was done for 12 h at 39°C. Aflatoxin degradation assay was performed by ELISA. Means with different superscripts $^{(a,b)}$differ significantly ($p < 0.05$).

Since it is commonly accepted bacteria in the rumen are responsible for the metabolism of whatever plant matter is consumed by an animal, investigations were performed to study if the observed difference between native and domestic livestock was due to rumen microbes. In a study by Jones and Megarrity [11], resistance to *Leucaena* toxicosis was successfully conveyed from Hawaiian goats to Australian cattle by transferring whole rumen fluid.

Rumen fluid also displayed different levels of degradation when applied to various treatments, though not large enough to be significant. The reason why rumen fluid filtered by a Millipore (0.45 μm) filter showed a numerically lesser degradation when compared to cheese cloth-filtered rumen fluid might be due to the Millipore filter pore size and the types of microbes limited by this treatment. Indeed microbe type may be a factor as Kiessling *et al*. [14] reported protozoa were more active than bacteria in the degradation of OTA, ZON and T-2 toxin .

No degradation was observed in autoclaved rumen fluid in the present study. This is most likely attributed to the destruction of live microbes and enzymes in autoclaved rumen fluid, therefore indicating the role of microbes in toxin degradation.

The time required for the biotransformation of toxins which enter the body through the digestive tract is also important, as some toxins are degraded within a short time and others need longer. Zearalenone toxin was degraded by bovine rumen fluid by an average of 37.5% after 48 h of incubation [12]. In our study, degradation of aflatoxin could be seen after incubation of 3 h or more, in both species. Several studies suggest feeding time influences the biotransformation of mycotoxins entering the digestive tract. In fact, the microbial population as well as the metabolic activity of the microbes increases at specific times after feeding, leading to higher mycotoxin degradation. Indeed, Keisseling *et al*. [14] observed the capacity to degrade Ochratoxin A decreased after feeding yet was restored by the next feeding time. However, our study showed rumen fluid collection time did not significantly affect AFB1 degradation in the two species.

Some reports indicate mycotoxin effects were moderated by different environmental factors, stress, animal's physiological ability and their preference for food [25]. Though the experimental steers and goats in our study were provided identical feed and environmental conditions, the possibility for differing food preferences might have influenced the rumen fluid components and bacterial population, thereby leading to differences in aflatoxin degradability. In addition, not only the feeding time but also the type of feed influenced degradation. With high-concentrate diets, ability to degrade OTA falls by 20% [14]. Similar observations in our study showed AFB1 degradation was about 25% when goats were fed a roughage:concentrate mixture of 60 : 40, compared to an average of about 50% when fed a 80 : 20 mixture. Possibly responsible is the influence feed could have on the number and types of microbes residing in the rumen ecosystem. Our comparative study on aflatoxin degradation, by feeding rice straw or timothy hay as roughage sources to three goats, showed rumen fluid obtained from timothy fed animals demonstrated better aflatoxin degradation, although not significantly higher. Not surprising, the nutritional quality of timothy is better than that of rice straw. Obviously microbes can use the feed source from the host animal for their own survival easiest with quality feed. Moreover, a higher number of microbes will often increase metabolic activity, leading to higher degradation of aflatoxin. However, individual differences in aflatoxin degradation existed among the three goats. This may be because individual animals have unique physical abilities, organ sizes, functions, sensory abilities and microbial populations.

In conclusion, our experimental findings show rumen microbes from Korean native goats demonstrated higher AFB1 degradability compared to Holstein steers. We observed AFB1 degradation in rumen fluid was influenced by animal species and type of feed fed to the animals. Individual animals and to a certain extent, the feeding and incubation time also contributed. The findings from this study furthers our research in selecting species as potential rumen fluid donors for the isolation of bacteria having aflatoxin degrading ability.

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