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RESEARCH ARTICLE

Efficacy of Modified Yeast Extract and HSCAS Containing Mycotoxin Adsorbent on Ruminal Binding Characteristics of Various Aflatoxins [1]

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Summary

The objective of this experiment was to determine the ruminal binding characteristics of modified *S. cerevisiae* extract and hydrated sodium calcium aluminosilicate (HSCAS) containing mycotoxin adsorbent (MA) against various aflatoxins in an *in vitro* study. A certified aflatoxin mixture (B1, G1, B2, G2) in a liquid form was mixed with ruminal *in vitro* medium providing the final concentrations of 6 ng/ml aflatoxin B1 (AFB1), 6 ng/ml aflatoxin G1 (AFG1), 1.5 ng/ml aflatoxin B2 (AFB2), and 1.5 ng/ml aflatoxin G2 (AFG2). Treatments were: 1) aflatoxin mixture + distilled water (Control); 2) aflatoxin mixture + rumen fluid (AR); and 3) aflatoxin mixture + MA (6 mg) + rumen fluid (AMAR). After various incubation time points (0, 3, 6, 12, 24 h) at 39°C, aflatoxin concentrations in ruminal medium were detected with HPLC. Although AFB1 concentration at 0 h was 6 ng/ml, it was reduced to 2.50 and 1.68 ng/ml in Control, 0.86 and 0.50 ng/ml in AR, and 0.34 and 0.20 ng/ml in AMAR treatments at 3 and 12 h, respectively (P<0.001). In addition, AFB1 concentration in AMAR treatment was in a steady-state condition after 3 h of incubation compared to Control and AR treatments where AFB1 concentration became stabilized after 12 h of incubation. A similar type of binding pattern was also observed for AMAR treatment in ruminal incubation of AFB2. In addition, the concentrations of both AFG1 and AFG2 were in a steady-state condition for AR (0.67 and 0.48 ng/ml) and AMAR (0.46 and 0.38 ng/ml) treatments after 12 h of ruminal incubation. The binding capability of the MA on AFG1 and AFG2 was always in favor of AMAR treatment at all time points. There was no treatment effect on ruminal *in vitro* gas production across all treatments, averaging 53.5 ml at 24 h. Results indicate that the MA can help binding the studied aflatoxins and reducing their concentrations in the rumen before they enter into the bloodstream.

Keywords: *Modified yeast extract, HSCAS, Aflatoxin, Ruminal binding*

Modifiye Maya Ekstraktı ve HSCAS İçeren Mikotoksin Bağlayıcının Rumende Aflatoksinleri Bağlama Etkinliği

Özet

Bu araştırma, modifiye *S. cerevisiae* ve hidrate sodyum kalsiyum alüminosilikat (HSKAS) içeren mikotoksin bağlayıcının (MB) çeşitli aflatoksinlere karşı *in vitro* bir çalışmada ruminal bağlanma etkinliğini belirlemeyi amaçlamıştır. Sıvı formdaki sertifikalı aflatoksin karışımı (B1, G1, B2, G2) rumen sıvısıyla *in vitro* ortamda karıştırılarak final konsantrasyonları 6 ng/ml aflatoksin B1 (AFB1), 6 ng/ml aflatoksin G1 (AFG1), 1.5 ng/ml aflatoksin B2 (AFB2), ve 1.5 ng/ml aflatoksin G2 (AFG2) olacak şekilde hazırlanmıştır. Muamele grupları: 1) aflatoksin karışımı + distile su (Kontrol); 2) aflatoksin karışımı + rumen sıvısı (AR); ve 3) aflatoksin karışımı + MB (6 mg) + rumen sıvısı (AMBR) olarak planlanmıştır. Rumen sıvısındaki aflatoksin konsantrasyonları 39°C'deki inkübasyon zamanlarından (0, 3, 6, 12, 24 saat) sonra HPLC ile tayin edilmiştir. Her ne kadar AFB1 konsantrasyonu 0. saatte 6 ng/ml iken, bu konsantrasyon 3. ve 12. saatlerde kontrol grubunda sırasıyla 2.50 ve 1.68 ng/ml'ye, AR grubunda 0.86 ve 0.50 ng/ml'ye, AMBR grubunda ise 0.34 ve 0.20 ng/ml'ye düşmüştür (P<0.001). Ayrıca AMBR grubundaki AFB1 konsantrasyonu, Kontrol ve AR gruplarıyla karşılaştırıldığında inkübasyonun 3. saatinden sonra sabit değerlere ulaşmıştır. Ancak AFB1 konsantrasyonundaki sabit değerlere ulaşma Kontrol ve AR gruplarında inkübasyonun 12. saatinden sonra gerçekleşmiştir. Benzer tipteki bağlanma şekli AMBR muamelesinde AFB2'nin ruminal inkübasyonunda da gözlenmiştir. Ayrıca AFG1 ve AFG2 konsantrasyonları, AR (0.67 ve 0.48 ng/ml) ve AMBR (0.46 ve 0.38 ng/ml) gruplarında ruminal inkübasyonun 12. saatinden sonra sabit değerlere ulaşmıştır. Kullanılan MB'nin AFG1 ve AFG2 üzerindeki bağlama kabiliyeti ruminal inkübasyonun bütün zaman dilimlerinde AMBR grubu lehine olmuştur. Muamele gruplarının rumen *in vitro* gaz üretimi üzerine bir etkileri gözlenmemiş ve 24. saatte ortalama 53.5 ml olmuştur. Sonuçlar MB'nin çalışılan aflatoksinleri rumen ortamında kan dolaşımına girmeden önce bağlama yeteneğine yardımcı olabileceğini ve konsantrasyonlarını düşürebileceğini göstermiştir.

Anahtar sözcükler: *Modifiye maya ekstraktı, HSKAS, Aflatoksin, Ruminal bağlanma*

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INTRODUCTION

Aflatoxins are secondary metabolic substances of various *Aspergillus* spp. *(A. flavus, A. nominus, A. parasiticus*). Based on its composition and toxicity, AFB1 is the most important aflatoxin followed by AFG1, AFB2 and, AFG2 [1]. High doses of aflatoxins in food and animal feed often cause acute toxic effects that can result in significant health problems and economic losses. In the presence of low doses, aflatoxins may result in hepatocarcinogenic, mutagenic, and teratogenic effects along with immune system suppression. All livestock, birds and many species of fish are susceptible to the effects of aflatoxins [2]. As a result, all foods of animal origin (meat, milk) can be affected by aflatoxin contamination [3,4].

Much research has focused on preventing aflatoxin contamination in animal feed by using mycotoxin binding agents to control the harmful effects of aflatoxin on animal health and the transference of the aflatoxins into meat and milk. Because these agents are not subject to any changes in an animal's digestive tract, the toxic effects of mycotoxins on animals along with their passage into animal products via absorption from the digestive tract are slowed down when they are included in the diet [5]. Mycotoxin binding agents with the most potential include activated carbon, aluminosilicates (clay, bentonite, montmorillonite, zeolite, hydrated sodium calcium aluminosilicate (HSCAS)), non-digestible complex carbohydrates (the cell walls of bacteria and yeast), and synthetic polymers, such as cholestyramine [6].

Hydrated sodium calcium aluminosilicate, produced from natural zeolite, has high adsorbing characteristics against mycotoxins. When it is mixed with aflatoxin contaminated animal feeds, it can have a protective effect against the development of aflatoxicosis in animals [7]. Yeast cell wall extracts have been proposed as another alternative source of mycotoxin adsorbent. They do not reduce the nutritional value of the feed or create a harmful effect on the environment [8]. In addition, recent research demonstrated that using yeast cell wall extracts in lactating dairy cattle's diet contaminated with aflatoxins reduced the AFM1 excretion in raw milk significantly [9].

Since the main site of aflatoxin biodegradation in the animal's metabolism is still being debated, results from the ruminal biodegradation of aflatoxins in ruminant animals are contradictory. Engel and Hagemeister [10] reported a significant reduction in AFB1 concentration when incubated in ruminal fluid. However, Kiessling et al.[11] could not detect any reduction of AFB1 in inoculated rumen fluid. Auerbach et al.[12] proposed that AFB1 inoculation in ruminal fluid and its *in vitro* incubation may produce secondary metabolites (aflatoxicol) and cause incomplete biodegradation. Kiessling et al.[11] also indicated that mycotoxins are less toxic in ruminants than other animals due to a partial biodegradation in the rumen, but some biodegradation products may still remain toxic.

Our objective in this study was to determine the *in vitro* ruminal binding characteristics of modified *S. cerevisiae* extract and HSCAS containing mycotoxin adsorbent (MA) at certain incubation time points against various aflatoxins. In addition, ruminal *in vitro* gas production method was used for detecting the effects of those aflatoxins and their combinations with MA on ruminal fermentation.

MATERIALS and METHODS

Sample Preparation

A certified aflatoxin mixture (Biopure L09034A) having a total concentration of 5 µg/ml (2 µg/ml AFB1, 0.5 µg/ml AFB2, 2 µg/ml AFG1, and 0.5 µg/ml AFG2) in liquid form was used in the experiment. A mixture of modified *S. cerevisiae* extract and HSCAS was used as a mycotoxin adsorbent (MycoPurge®; MA). Fresh ruminal fluid from a slaughtered cow was immediately brought to the laboratory, and then filtered and mixed with artificial saliva [13]. Thirty ml of ruminal samples (10 ml rumen fluid + 20 ml artificial saliva) were incubated in 50 ml of tightly sealed bottles at 39°C for 24 h [13]. Aflatoxin and *in vitro* gas production analyses were performed in five replicates at 0, 3, 6, 12, and 24 h for each treatment. Treatment groups were: 1) aflatoxin added distilled water (Control), 2) aflatoxin added ruminal fluid (AR), and 3) aflatoxin and MA added ruminal fluid (AMAR). For *in vitro* gas production measurements, treatments were: 1) aflatoxin added ruminal fluid (AR), 2) MA added ruminal fluid (MAR), and 3) aflatoxin and MA added ruminal fluid (AMAR). Except for the Control group, 1 g of wheat starch was added into each bottle prior to incubation to act as a substrate for ruminal fermentation. A mixture of aflatoxins (6 ng/ml AFB1, 6 ng/ml AFG1, 1.5 ng/ml AFB2, 1.5 ng/ml AFG2) was added into each *in vitro* medium for Control, AR, and AMAR groups. In addition, 6 g of MA was added into each medium for MAR and AMAR groups.

Analyses of Ruminal Aflatoxins and in vitro Gas Production

The method used for aflatoxin determination in ruminal fluid was based on the principle of HPLC-immunoaffinity column [14]. Twenty ml of treatment ruminal medium was taken from the bottles and diluted with 80 ml of distilled water, which was then filtered through filter paper (Whatman #4) to separate the particulate fraction. This filtered sample was then again re-filtered through a microfiber glass filter to finalize the volume of 10 ml and introduced to immunoaffinity column (Vicam, Afla Test-P). The bound aflatoxins to those immuno-affinity columns were then treated with 1 ml of methanol and distilled water, respectively, to separate the respective aflatoxins. This elute was transferred into a vial and vortexed before injecting into the HPLC. The HPLC conditions were as follows: wave-length of Ex: 360 nm, Em: 440 nm; fluorescein detector; ODS-2 (C18 -250 mm-5µm-4,6 mm) column; 25°C column temperature; 1 ml/sec pump flowing rate; mobile phase of distilled water/acetonitrile/

methanol (550/200/300 v/v/v); injection volume of 100 µL. Recoveries in the method were 91, 85, 87, and 55% for AFB1, AFG1, AFB2, and AFG2, respectively. In addition, the detection limits were 0.06, 0.06, 0.04, and 0.08 ng/ml for AFB1, AFG1, AFB2, and AFG2, respectively.

Statistical Analysis

Statistical analysis of the data from aflatoxins in ruminal fluid was performed as the PROC MIXED procedure for repeated measures of SAS (ver. 8.01) with time as the repeated measure using a model that included treatment, time, and the treatment × time interaction. *In vitro* gas production data was calculated based on the model described by Orskov and McDonald [15]. In the model, cumulative gas production data were fitted to the following equation: $y= a+b (1-exp^{-ct})$ where, y is the gas production at time t; a is the gas production from the rapidly soluble fraction (ml); b is the gas production from the insoluble fraction (ml); c is the gas production rate constant; a+b is the potential gas production (ml); and t is the incubation time (h). The data obtained from *in vitro* gas production was analyzed by PROC GLM procedure of SAS (ver. 8.01). The LSD multiple comparison test was performed for treatment mean differences.

RESULTS

Concentrations of *in vitro* incubation of aflatoxin mixture with ruminal fluid and the MA at various time points are presen ted in *Fig. 1*. Initial AFB1 concentration in the Control group was 6 ng/ml. A significant reduction in AFB1 concentration was detected at 3 h of incubation relative to their initial concentration by 58.3 (2.50 ng/ml), 85.7 (0.86 ng/ml), and 94.3% (0.34 ng/ml) for Control, AR, and AMAR treatments, respectively (P<0.001). Similarly, this trend was also observed at 12 h of incubation by 72.0 (1.68 ng/ml), 91.7 (0.50 ng/ml), and 96.7% (0.20 ng/ml) for Control, AR, and AMAR treatments, respectively (P<0.001). However, a decrease in AFB1 concentration among treatments was different across the incubation time points, where it no longer declined and became stable after 12 h of incubation for Control and AR compared to AMAR treatment after 6 h of incubation. It can be concluded that reduction in AFB1 concentration was much faster for AMAR than the Control and AR treatments due to the MA in this treatment. Repeated measures of the data analyses also indicated that 3 h of ruminal incubation results were significantly different than 6 (P= 0.01) and 12 h (P = 0.02) but not from 24 h (P = 0.45) of incubation (treatment by time interaction). A significant reduction in AFB2 concentration was detected at 3 h of incubation by 28.7, 70.0, and 82.0% for Control, AR, and AMAR treatments, respectively (P<0.001). However AFG1 concentration in the *in vitro* medium reduced at 12 h by 87.2 (0.77 ng/ml), 88.8 (0.67 ng/ml), and 92.3% (0.46 ng/ml) for Control, AR, and AMAR treatments, respectively (P<0.001). Relative to the initial concentration of AFG2 in the *in vitro* medium, both AR and AMAR treatments showed a significant AFG2 concentration reduction (0.48 and 0.38 ng/ml) at 12 h compared to the Control (0.60 ng/ml). Data also state that ruminal aflatoxin (B1, G1, B2, G2) binding capability were unchanged after 6 h of incubation for both AR and AMAR treatments (refer to *Fig. 1*).

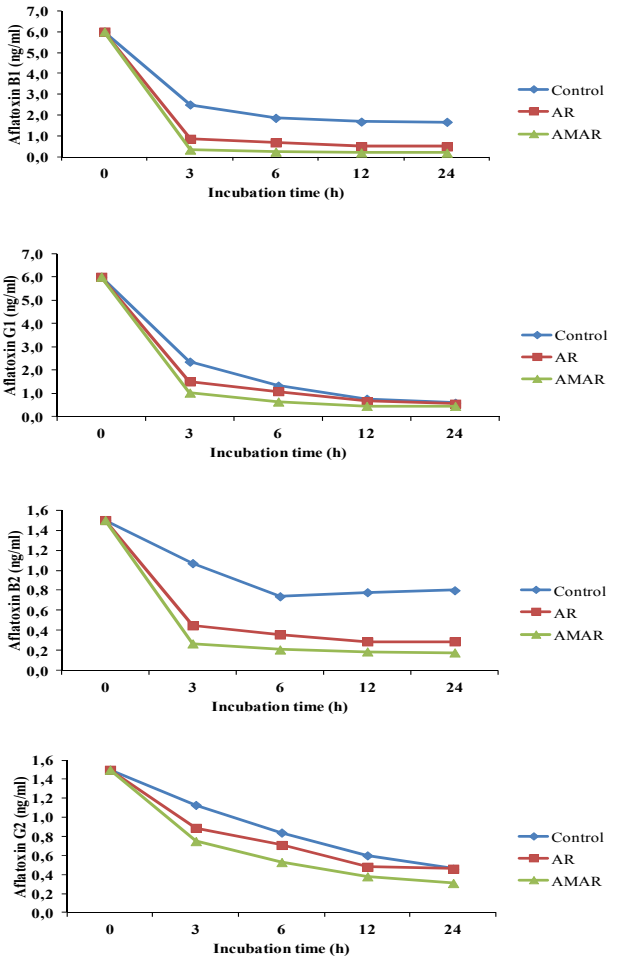

Fig 1. Concentrations (ng/ml) of aflatoxins (B1, G1, B2, G2) incubated with ruminal fluid (AR) and the mycotoxin adsorbent (AMAR) *in vitro*

Şekil 1. Rumen sıvısıyla (AR) ve mikotoksin bağlayıcı (AMAR) ile *in vitro* inkübe edilmiş aflatoksinlerin (B1, G1, B2, G2) konsantrasyonları (ng/ml)

Pooled standard error of mean: 0.04, 0.06, 0.02, and 0.03 for AFB1, AFG1, AFB2, and AFG2, respectively

In vitro ruminal gas production data from the respective treatments are presented in *Table 1*. The aflatoxin mixture, MA addition or their combinations did not have an effect on *in vitro* ruminal gas production at any incubation time points.

DISCUSSION

The Control, AR, and AMAR treatments showed a linear reduction of AFB1, AFG1, AFB2, and AFG2 up to 12 h of incubation, then the reductions were asymptotic. In Control treatment, all aflatoxin concentrations were subjected to reduction in the presence of water and heat of incubation (39°C). For this treatment, AFB1 and AFG1 reductions within 3 h of incubation were 58.3 and 60.7%, respectively. Doyle et al.[16] indicated that the rising moisture content of oil seed

Table 1. *In vitro gas production of ruminal fluid incubated with aflatoxins (B1, G1, B2, G2) and mycotoxin adsorbent at various time points*
Tablo 1. *Çeşitli zaman aralıklarında aflatoksinler (B1, G1, B2, G2) ve mikotoksin bağlayıcı ile inkübe edilen rumen sıvısında in vitro gaz üretimi*

Incubation Time (h)	Treatments			SEM*
	Aflatoxins (B1, G1, B2, G2) + Ruminal Fluid	Mycotoxin Adsorbent + Ruminal Fluid	Aflatoxins (B1, G1, B2, G2) + Mycotoxin Adsorbent + Ruminal Fluid	
	in vitro gas production (ml)			
3	3.5	2.2	2.8	0.7
6	10.6	10.0	9.9	0.5
12	25.6	26.5	26.7	0.8
24	52.6	54.7	53.2	2.2

**Pooled standard error of mean*

meals with a constant product temperature resulted in a significant reduction of aflatoxins. This study indicated that the presence of moisture and heat in feed might help reduce aflatoxin concentrations. In this experiment, even a very low *in vitro* incubation temperature of 39°C caused significant reduction in aflatoxin concentrations. In AR treatment, this reduction was much greater, possibly because of the effect of ruminal microflora in addition to the temperature of the inoculum for degrading the aflatoxins. Although data presenting the susceptibility of rumen microflora to aflatoxins are contradictory, Engel and Hagemeister [10] found that 40% of the dietary AFB1 was degraded in the rumen. In addition, they also demonstrated that only 2-5% of the AFB1 reached the intestines of cows, thus showing the efficiency of rumen metabolism for aflatoxin degradation [10,17]. On the contrary, Kiessling et al.[11] tested the metabolism of AFB1 by intact rumen fluid, rumen protozoa and bacteria *in vitro*. They found that AFB1 was not degraded by rumen microflora. There was a slight reduction in AFB1 concentration that occurred only within 30 min of incubation, and then no further reduction was evident. Westlake et al.[18] also found that 10 µg/ml of AFB1 in an *in vitro* rumen medium did not cause any toxic effect on four strains of *Butyrivibrio fibrisolvens*, where the remaining AFB1 concentration after incubation to stationary phase was 95%. They proposed that the rumen microbial activity was solely responsible for aflatoxin degradation, and that dietary composition of the ruminant animal could play an important role in conferring aflatoxin resistance. Another possible explanation of no aflatoxin detection in the rumen is the conversion of those aflatoxins into their metabolites, such as aflatoxicol [19]. Except AFG1 and AFG2, both AFB1 and AFB2 concentrations decreased significantly by 71 and 64%, respectively at 24 h of incubation in the ruminal fluid alone compared to the Control treatment. Similarly, Upadhaya et al.[20] tested the AFB1 degradation in ruminal fluids of cattle and goats *in vitro*, and found a significant reduction of AFB1 by 14 and 25%, respectively, at 3 h of incubation.

There have been many aflatoxin adsorbent agents proposed for controlling the aflatoxicosis in livestock animals. Mineral clays, and more recently, modified *S. cerevisiae* extracts are used commonly. Phillips et al.[21] stated that HSCAS had a significant aflatoxin binding capability both *in vivo* and *in vitro* by 80% in the digestive tract of livestock. In addition, Spotti et al.[22] confirmed that 25 mg of HSCAS bound successfully to 200 ng of AFB1 in both rumen fluid and water within 2 h of *in vitro* incubation at 39°C. It has been shown that modified *S. cerevisiae* extracts would be able to bind to aflatoxins by approximately 75% within a wide pH spectrum [23]. Moschini et al.[24] studied two commercial aluminosilicates and a yeast cell wall derivative as sequestering agents to verify their binding capacity to AFB1 either in water or in ruminal fluid. In contrast to the findings in this study, their results indicated that the yeast cell wall derivative had a lower binding efficacy in both water (36%) and ruminal fluid (21%) compared to aluminosilicates (84 and 99% in water and ruminal fluid, respectively). Still other research found that the components of *S. cerevisiae* cell wall after their chemical modification were able to bind up to 95% of the aflatoxins [25]. Diaz et al.[26] also found a 96.6% binding capability of 1.11 g of yeast cell wall (glucomannan) against 5 µg/ml of AFB1 in an *in vitro* experiment.

As the *in vitro* incubation period extends through 24 h, concentrations of all tested aflatoxins are reduced in both AR and AMAR treatments. Although Fink-Gremmels [27] proposed that various mycotoxins possess anti-bacterial, anti-protozoal and anti-fungal activity, and as a result cause adverse effects on rumen environment, ruminal microflora could not possibly be affected by the aflatoxin presence in AR and AMAR treatments in this experiment. This could have been due to the low aflatoxin concentration (6 ng/ml) used in the study. No *in vitro* gas production difference observed in all treatments also supported this finding *(Table 1)*.

In conclusion, results indicate that aflatoxins can be eliminated by the heat of the incubation medium along with microbial degradation. In addition, MA having a combination of modified *S. cerevisiae* extract and HSCAS can help binding those aflatoxins and reducing their concentrations in the rumen within short period of time (about 6 h) before they enter into the bloodstream. This type of binding capability of modified yeast extract and HSCAS containing mycotoxin adsorbent with the aid of rumen microorganisms may also protect ruminants from feed contaminated aflatoxicosis.

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