Short communication: Effects of a physiologically relevant concentration of aflatoxin B₁ with or without sequestering agents on in vitro rumen fermentation of a dairy cow diet

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ABSTRACT

Aflatoxin is a potent carcinogen commonly found in animal feeds that can impair rumen fermentation at high concentrations; however, its effects at physiologically relevant concentrations are unknown. This study examined the effects of aflatoxin B₁ (AFB₁), with or without bentonite clay (CL) and *Saccharomyces cerevisiae* fermentation product (SCFP)-based sequestering agents on in vitro rumen fermentation and digestibility of a dairy cow TMR. Corn silage-based TMR (0.5 g, 17.3% crude protein and 1.67 Mcal/kg of net energy for lactation) was incubated in a rumen fluid-buffer inoculum (1:2 ratio; 50 mL) with the following treatments: (1) no additives (control); (2) control + 0.75 µg/L AFB₁ (T); (3) T + 80 mg/L sodium bentonite clay (CL; Astra-Ben-20, Prince Agri Products Inc., Quincy, IL); or (4) CL + 14 mg/L SCFP (CL+SCFP; Diamond V, Cedar Rapids, IA). Ruminal fluid was collected 3 h after the morning feeding from 3 cannulated cows fed the same TMR, and rumen fluid from individual cows was used to prepare separate inocula. Each treatment was incubated in duplicate at 39°C for 0, 4, 8, 16, and 24 h in each of 3 runs. Adding T reduced total volatile fatty acid (VFA) concentration after 4 and 8 h and molar proportion of propionate after 4 and 24 h of incubation relative to control. Adding sequestering agents (CL and CL+SCFP) with T did not affect total VFA concentration after 4 or 8 h, but increased total VFA after 16 h and tended to increase molar proportion of

propionate after 24 h compared with T. At 24 h, T had lower DM digestibility and higher NH₃-N concentration compared with the control. Thus, AFB₁, even at very low concentration (0.75 µg/L), had detrimental effects on rumen fermentation and subsequently DM digestibility of the TMR. Adding sequestering agents did not prevent negative effects of T on rumen fermentation within 8 h of incubation; however, sequestering agents were effective after 16 h of incubation.

Key words: aflatoxin, sequestering agent, bentonite clay, rumen fermentation

Short Communication

Several in vitro and in vivo studies have investigated the effects of aflatoxin on ruminal fermentation and the growth of selected ruminal microorganisms (Mertens, 1979; Sinha and Arora, 1982; Jiang et al., 2012). Mertens (1979) suggested that aflatoxin may decrease growth and performance of animals by disrupting the growth and function of ruminal microorganisms. Sinha and Arora (1982) reported that cotton cellulose treated with 100, 250, 500, 1,000, and 1,250 µg/L aflatoxins had cellulose disappearance rates of 43, 21, 21, 16, and 16%, respectively. In addition, microbial protein synthesis was reduced from 48 (control) to 31, 11, 10, 9, and –2.5 mg/20 mL of inoculum, respectively, with increasing concentration of aflatoxin. Similarly, Westlake et al. (1989) reported that 1,000 and 10,000 µg/L aflatoxin B₁ (AFB₁) inhibited in vitro DM degradation of alfalfa hay by 50 and 67%, respectively. Jiang et al. (2012) reported lower gas production and VFA concentration with 320, 640, 960 µg/L AFB₁ when alfalfa or ryegrass was used as substrate. However, measured aflatoxin concentrations in feeds and livestock are often much lower. A 3-yr survey from 2009 to 2011 on worldwide occurrence of mycotoxins reported aflatoxin contamination in 33% of total of 7,049 feed samples (corn, soybeans, soybean meal, wheat, and dried distillers grains

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with solubles; Rodrigues and Naehrer, 2012) with an average concentration of 29 µg/kg from feeds in North America. An in vivo study by Sulzberger et al. (2017) reported an AFB₁ concentration of 0.20 µg/L in the rumen of dairy cows challenged with 100 µg of AFB₁/kg of diet DM. Hence, studies that reported negative effects of aflatoxin on ruminal fermentation are based on high doses (100 to 10,000 µg/L in the inoculum; Sinha and Arora, 1982; Westlake et al., 1989; Jiang et al., 2012), which are not likely to occur in the rumen.

The objective of this study was to examine whether a physiologically relevant concentration of aflatoxin with or without sequestering agents based on bentonite clay and *Saccharomyces cerevisiae* fermentation product (SCFP) would affect in vitro ruminal fermentation of a dairy cow diet over time after different incubation durations. Bentonite clay and SCFP were selected as sequestering agents because they reduced milk AFM1, and SCFP increased milk production compared with cows dosed with AFB₁ in our previous dairy cow study (Jiang et al., 2018). We hypothesized that a low concentration of aflatoxin (0.75 µg/L) would have detrimental effects on in vitro ruminal fermentation and that, simultaneously, supplementing sequestering agents would prevent such negative effects on fermentation.

The ruminal fluid collection protocol was approved by the University of Florida Institutional Animal Care and Use Committee (study no. 201509099). Three ruminally cannulated cows were adapted to a corn silage-based TMR that contained a 44:56 forage-to-concentrate ratio. The CP, NDF, ADF, and starch concentrations of the diet were 17.3, 33.6, 20.5, and 20.7% of DM, respectively. The in vitro procedure was conducted according to the method described by Romero et al. (2015). Treatments were (1) no additives (control); (2) control + 0.75 µg/L AFB₁ (T); (3) T + 80 mg/L bentonite clay (CL); and (4) CL + 14 mg/L SCFP (CL+SCFP). Previous studies showed that SCFP, a mixture of yeast culture medium and yeast cells, can alter gut microbiota, improve gut morphology, and bind aflatoxin to its cell wall as well as reduce inflammatory responses and ultimately improve performance of cows (Jensen et al., 2015; Xiao et al., 2016; Gonçalves et al., 2017). The combination of CL and SCFP was selected as a fourth treatment in this experiment to explore the synergistic effects of bentonite clay, which binds aflatoxin, with SCFP, which may also bind aflatoxin but potentially also improves rumen fermentation and animal performance by altering gut morphology and microbiota and reducing inflammatory responses. One milligram of AFB₁ powder was purchased (Sigma-Aldrich, St. Louis, MO), dissolved in 1 mL of methanol, and further diluted in 99 mL of double-distilled water.

Subsequently, 1 mL of the mixture was further diluted in 99 mL of distilled water to make a 0.1 µg/mL AFB₁ solution. Exactly 375 µL of AFB₁ solution was added to treatments T, CL, and CL+SCFP to provide 0.75 µg/L AFB₁ at final concentration. In particular, we used a concentration similar to the average concentration (63 µg/kg of DM) of AFB₁-positive feed samples reported from a comprehensive global farm survey (Rodrigues and Naehrer, 2012). This concentration of AFB₁ (75 µg/kg of feed DM) results in 0.75 µg/L in the ruminal inoculum, which is much lower than the doses (100 to 10,000 µg/L in the inoculum) that have been used in previous studies (Westlake et al., 1989; Jiang et al., 2012, 2014). Exactly 200 µL of clay (20 mg/mL) suspension solution was added to CL and CL+SCFP tubes, and an additional 50 µL of SCFP (14 mg/mL) suspension solution was added to CL+SCFP tubes before adding the 50 mL of inoculum. All treatments contained the same amount of double-distilled water and methanol-water mixture containing no AFB₁. Ruminal fluid from 3 cows was collected 3 h after morning feeding and filtered through 4 layers of cheesecloth. The filtered ruminal fluid from individual cows was used to prepare separate inocula with 2:1 ratios of anaerobic medium to rumen fluid (Adesogan et al., 2005). The anaerobic media were prepared according to Goering and Van Soest (1970). The inoculum was kept at 39°C in a water bath with continuous infusion of CO₂ before in vitro inoculation. The TMR fed to the donor cows was used as a substrate after grinding it to pass through a 1-mm screen using a Wiley mill (A. H. Thomas, Philadelphia, PA). Approximately 0.5 g of ground substrate was weighed into each tube, and the tubes were transferred to an incubator set at 39°C. After incubating for 0, 4, 8, 16, and 24 h, tubes were placed on ice to stop the fermentation in each of 3 experimental runs. Samples from 0, 4, 8, and 16 h were added, with 50 µL of 50% sulfuric acid, and centrifuged at 7,974 × g for 15 min. The supernatant was stored at -20°C for VFA analysis. Samples taken after 24 h of incubation were used to measure pH using a digital pH meter (Accumet AB15, Fisher Scientific, Hampton, NH) and determine DM digestibility (DMD) by filtering through filter paper and drying residues in a forced-air oven at 55°C for 48 h. Later, 40 mL of filtrate was mixed with 40 µL of 50% sulfuric acid, centrifuged, and used for analysis of VFA, lactic acid, and NH₃-N concentrations. Volatile fatty acids and lactic acid were determined using an HPLC system (Hitachi, L2400, Tokyo, Japan). The column (Aminex HPX-87H, Bio-Rad Laboratories, Hercules, CA) was set with a 0.7 mL/min flow rate at 45°C, using 0.015 M H₂SO₄ as mobile phase. Ammonia-N was measured via colorimetric quantification of N content using

Figure 1. Effects of supplementing sequestering agents based on sodium bentonite clay and *Saccharomyces cerevisiae* fermentation product (SCFP) with or without aflatoxin B₁ (AFB₁) on total VFA concentration during in vitro fermentation. Control treatment = no additives; T = control + 0.75 µg/L AFB₁; CL = T + 80 mg/L bentonite clay; CL+SCFP = CL + 14 mg/L SCFP. T had lower ($P < 0.05$) total VFA concentration at h 4 and 8 relative to control. Sequestering agent treatments (CL and CL+SCFP) had higher ($P < 0.05$) total VFA compared with T at h 16. Treatments have similar VFA concentrations at 24 h. Data are LSM ± SEM.

an autoanalyzer (Technicon, Tarrytown, NY) according to an adaptation of Noel and Hambleton (1976).

Data were analyzed separately for each time point. The GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC) was used to analyze the data, using the following model:

$$Y = \mu + \text{Trt}_i + R_j + C_k + (C \times R)_{jk} + e_{ijk},$$

where μ is the overall mean; Trt_i is the fixed effects of treatment; R , C , and $(C \times R)_{jk}$ are the random effects of run, cow, and their interaction; and e_{ijk} is the residual error. Preplanned nonorthogonal contrasts were used to test the differences between the control treatment and T, T and the sequestering agent treatments (CL and CL+SCFP), and CL and CL+SCFP. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

Concentrations of total VFA are shown in Figure 1. Adding AFB₁ reduced ($P < 0.05$) the concentration of total VFA at 4 and 8 h, suggesting detrimental effects of AFB₁ at 0.75 µg/L on ruminal fermentation. This may be due to the antimicrobial property of aflatoxin, as it was reported to inhibit DNA and RNA synthesis through binding with N7 guanine on DNA strands (Butler and Neal, 1977; Upadhyaya et al., 2010). Some

early studies suggested that aflatoxin could inhibit the growth of *Lactobacillus bulgaricus*, *Exiguobacterium aurantiacum*, and *Escherichia coli* by impairing the activity of DNA polymerase (Sinha and Arora, 1982). In addition, reduced microbial protein synthesis by aflatoxin was also reported (Sinha and Arora, 1982), further supporting the antimicrobial properties of aflatoxin.

Adding sequestering agents did not reverse the reductions in VFA production at 4 and 8 h but increased ($P < 0.05$) concentrations of total VFA at 16 h compared with T. An in vivo study by Sulzberger et al. (2017) detected lower AFB₁ concentrations in the rumen of cows fed clay during an aflatoxin challenge. Similarly, Spotti et al. (2005) reported complete binding of AFB₁ in rumen fluid after 2 h of supplementing a hydrated sodium calcium aluminosilicate, sepiolite, and a bentonite at a higher application rate of 35 g/L. However, our results indicated that adding 80 mg/L CL, with or without 14 mg/L SCFP, did not have beneficial effects on fermentation in the first 8 h but did after 16 h of incubation, indicating that adequate time is needed for a low concentration of sequestering agents to bind with AFB₁. This agrees with our previous in vivo study (Jiang et al., 2018), in which feeding bentonite clay did

not reduce milk AFM1 below the FDA action level (0.5 µg/kg) on the first day of the challenge but led to a 40% reduction in AFM1 on the second day.

Interestingly, at 16 h, the total VFA concentration in the Control and T treatments did not follow the general pattern of increasing over incubation time, for unknown reasons. Treatments resulted in similar concentrations of VFA at 24 h, which may be because sequestering agents had sufficiently bound to AFB₁, or because of reduced activity of aflatoxin at this later stage of fermentation, possibly due to degradation by ruminal microbes (Upadhaya et al., 2009).

The VFA molar proportion, lactate, pH, and NH₃-N concentrations are shown in Table 1. For unknown reasons, lactate concentration was lower ($P = 0.002$) with sequestering agents compared with T at 0 h. At 24 h, lactate was lower in T than in control ($P = 0.001$), which could be caused by the toxicity of AFB₁ to lactate-producing bacteria. Lactate tended to be higher ($P = 0.08$) in the sequestering agent treatments than in T at 24 h, suggesting that part of AFB₁ had bound to the sequestering agents, reducing their negative effects on lactate-producing bacteria. A similar trend was also observed in total VFA concentration. Compared with

Table 1. Effects of supplementing sequestering agents based on sodium bentonite clay and *Saccharomyces cerevisiae* fermentation product (SCFP) with or without aflatoxin B₁ (AFB₁) on in vitro rumen fermentation (molar %, unless otherwise stated)

Item	Treatment ¹				SEM	Contrast <i>P</i> -value		
	Control	T	CL	CL+SCFP		Control vs. T	T vs. CL and CL+SCFP	CL vs. CL+SCFP
0 h								
Acetate	54.1	56.3	53.8	53.7	2.80	0.02	0.004	0.87
Propionate	20.7	19.4	20.7	20.1	2.67	0.12	0.17	0.44
Butyrate	14.4	13.1	14.8	14.6	1.13	0.14	0.03	0.83
Valerate	2.13	1.96	1.98	2.29	0.73	0.70	0.64	0.48
Isobutyrate	7.63	8.21	7.30	8.04	1.72	0.22	0.19	0.13
Isovalerate	1.12	0.98	1.33	1.27	0.45	0.46	0.06	0.74
Lactate, mM	6.07	7.47	1.32	2.65	2.07	0.47	0.002	0.49
4 h								
Acetate	53.5	53.6	54.5	53.7	2.63	0.91	0.37	0.26
Propionate	24.7	24.0	23.6	24.3	4.31	0.18	0.93	0.15
Butyrate	15.2	15.0	14.7	15.2	1.52	0.74	0.90	0.31
Valerate	1.79	1.84	2.05	1.91	0.30	0.85	0.56	0.63
Isobutyrate	3.62	3.73	3.78	3.54	0.93	0.76	0.82	0.49
Isovalerate	1.20	1.82	1.39	1.26	0.35	0.005	0.008	0.54
Lactate, mM	0	0.11	0	0	0.17	0.16	0.11	1.00
8 h								
Acetate	52.7	52.7	53.2	53.8	2.82	0.98	0.27	0.39
Propionate	24.0	24.0	23.5	23.4	4.25	0.95	0.16	0.94
Butyrate	15.5	15.3	15.5	15.4	1.95	0.74	0.78	0.88
Valerate	2.77	2.84	3.16	2.68	0.58	0.83	0.80	0.19
Isobutyrate	3.50	3.28	3.01	3.16	0.65	0.65	0.64	0.75
Isovalerate	1.52	1.81	1.72	1.52	0.47	0.08	0.18	0.21
Lactate, mM	2.60	1.88	2.38	2.86	2.56	0.57	0.50	0.71
16 h								
Acetate	53.9	54.0	53.1	53.8	2.58	0.89	0.28	0.25
Propionate	22.8	22.9	22.6	22.8	3.48	0.75	0.47	0.51
Butyrate	14.8	15.0	14.7	14.2	1.67	0.70	0.21	0.19
Valerate	2.61	2.19	2.29	2.88	0.82	0.28	0.28	0.13
Isobutyrate	3.49	3.44	4.16	3.92	1.25	0.90	0.22	0.56
Isovalerate	2.35	2.49	3.10	2.45	0.53	0.69	0.31	0.05
Lactate, mM	9.80	6.94	9.58	9.48	4.83	0.15	0.13	0.96
24 h								
Acetate	53.5	53.2	53.8	53.6	1.96	0.75	0.55	0.89
Propionate	23.3	22.6	23.0	23.2	3.20	0.04	0.10	0.44
Butyrate	14.2	14.5	14.4	14.0	1.60	0.25	0.21	0.28
Valerate	2.81	3.80	3.37	3.38	0.60	0.02	0.23	0.97
Isobutyrate	3.40	3.09	2.59	2.67	0.43	0.48	0.23	0.86
Isovalerate	2.81	2.75	2.96	3.09	0.20	0.78	0.15	0.55
Lactate, mM	7.42	1.15	2.74	5.14	2.40	0.001	0.08	0.20
NH ₃ -N, mM/dL	11.8	12.4	11.8	12.4	0.80	0.01	0.15	0.03
pH	6.64	6.61	6.67	6.68	0.05	0.24	0.20	0.75

¹Control treatment = no additives; T = control + 0.75 µg/L AFB₁; CL = T + 80 mg/L bentonite clay; CL+SCFP = CL + 14 mg/L SCFP.

control, T had a greater ($P = 0.02$) molar proportion of acetic acid at 0 h, higher concentration of isovalerate at 4 h ($P = 0.005$) and 8 h ($P = 0.08$), and higher valerate ($P = 0.02$) and lower ($P = 0.04$) propionate proportions after 24 h of incubation. In general, 0.75 $\mu\text{g/L}$ AFB₁ shifted the fermentation toward acetate, valerate, and isovalerate at the expense of propionate, for unknown reasons. In contrast, Jiang et al. (2012) reported no effects of 0, 320, 640, or 960 $\mu\text{g/L}$ AFB₁ on propionate proportion in vitro, although they used alfalfa hay or ryegrass hay as substrate instead of the TMR used in the current study. More research is needed to understand why AFB₁ resulted in production of less propionate in the current study.

Compared with T, adding sequestering agents reduced ($P = 0.03$) the proportion of acetate and increased proportions of butyrate ($P = 0.03$) and isovalerate ($P = 0.06$) at 0 h. The molar proportion of isovalerate was lower at 4 h ($P < 0.01$), whereas the propionate proportion tended to be greater at 24 h ($P = 0.10$) with sequestering agents compared with T. The differences observed in the molar proportion of VFA with sequestering agents, compared with T, may be attributed to the ability of sequestering agents to bind AFB₁, reducing its negative effects on ruminal fermentation. Similar VFA concentrations in control and sequestering agent treatments further support this speculation. The

results in the present study are in agreement with a previous study (Jiang et al., 2014) that showed reduction in acetate and increase in propionate proportion with 0.1, 1, and 10 g/L smectite clay added in vitro with 1,000 $\mu\text{g/L}$ AFB₁ (Jiang et al., 2014).

No major difference was observed between CL or CL+SCFP on VFA profile, except that adding CL+SCFP reduced ($P = 0.05$) the proportion of isovalerate compared with adding CL after 16 h of incubation. This suggests that SCFP favored the growth of microorganisms other than isovalerate-producing bacteria. The similar VFA concentrations among CL, CL+SCFP, and control samples suggests that both CL and CL+SCFP reduced the shift in VFA profile caused by treatment T, possibly by binding with AFB₁.

The concentration of NH₃-N at 24 h was higher ($P < 0.05$) in T than in control samples (12.4 vs. 11.8 mM/dL) and in CL+SCFP than in CL samples (12.4 vs. 11.8 mM/dL). The increase in NH₃-N suggests that assimilation of NH₃-N might be reduced by T, which could be caused by impaired activity of NH₃-N-assimilating bacteria, perhaps by predominant species such as *Ruminococcus flavefaciens* strain C94, *Ruminococcus albus* strain 7, and *Eubacterium* strain B17 (Bryant and Robinson, 1963; Schaefer et al., 1980). Control and CL samples had similar NH₃-N concentrations, possibly due to binding of AFB₁ by CL. The reason for higher

Figure 2. Effects of supplementing sequestering agents based on sodium bentonite clay and *Saccharomyces cerevisiae* fermentation product (SCFP) with or without aflatoxin B₁ (AFB₁) on in vitro DM digestibility. Control treatment = no additives; T = control + 0.75 $\mu\text{g/L}$ AFB₁; CL = T + 80 mg/L bentonite clay; CL+SCFP = CL + 14 mg/L SCFP. T had lower ($P = 0.04$) DM digestibility than the control. No differences were found between T and sequestering agent treatments (CL and CL+SCFP) or between CL and CL+SCFP ($P > 0.10$). Data are LSM $\pm$ SEM.

NH₃-N in CL+SCFP compared with CL is unclear. The SCFP might have increased the activity or abundance of amino acid-utilizing bacteria and increased amino acid breakdown into pyruvate and free NH₃. For instance, relative abundance of *Butyrivibrio*, which can produce large amounts of NH₃ (Bladen et al., 1961), was increased in the rumen by supplementing live yeast to dairy cows (Jiang et al., 2017).

The DMD was reduced ($P = 0.04$; Figure 2) by T from 57.2 to 53.2%. The reduction in fermentation and DMD may be due to the toxic effects of aflatoxin on microorganisms, as discussed previously. Similar detrimental effects of aflatoxin on DMD have previously been reported (Westlake et al., 1989; Mojtahedi et al., 2013). Westlake et al. (1989) showed that 1,000 and 10,000 µg/L AFB₁ inhibited the degradation of alfalfa hay by 50 and 67%, respectively. Mojtahedi et al. (2013) reported reduction in total gas production and DMD with 300 to 900 µg/L AFB₁. Despite the much lower and physiologically realistic concentration of AFB₁ used in our study (0.75 µg/L in the inoculum) compared with other studies (1,000 and 10,000 µg/L, Westlake et al., 1989; 300 to 900 µg/L, Mojtahedi et al., 2013), reductions in DMD and VFA were still evident. These detrimental effects on ruminal fermentation are probably due to impaired activity of rumen microorganisms. Future research should focus on reducing aflatoxin contamination in feeds and developing more effective mitigation strategies to ensure optimal animal performance. Unexpectedly, CL+SCFP had similar effects to CL on total VFA concentration and DMD, suggesting that inclusion of SCFP did not facilitate greater binding of AFB₁ than CL alone in the rumen in the present study. Yet, in our companion study (Jiang et al., 2018), unlike CL alone, combining CL and SCFP tended to improve milk yield by 2 kg/d in dairy cows compared with T. This difference between in vivo and in vitro results may be because more time (25 d) was spent adapting rumen microbes to SCFP in the in vivo study than in the current in vitro study (24 h).

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