Effect of Fumonisin on Growth of Ruminal Bacteria in Batch Culture

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Abstract

Fumonisin (FMN) is a mycotoxin produced by Fusarium species and has been found in feeds fed to dairy cattle. The effects of FMN on ruminal bacterial growth and fermentation were measured with batch culture. Experimental feeds contained 0, 100 or 200 ppm FMN and were arranged as a completely randomized design (CRD). All feeds were incubated at 39°C with buffer and strained ruminal fluid. Culture optical density (OD) was measured to estimate microbial populations. The cultures were sampled at 0, 6, 12, 24 and 36 h to measure the fermentation end products, pH and OD. OD increased over time with no differences among treatments except at 12 h. At 12 h, OD was higher for cultures fed diets containing FMN at 100 (1.66) and 200 (1.62) ppm versus feed without FMN (1.41). Culture pH decreased over time and was not significantly different due to treatments over the entire study. Total concentration of VFA and concentrations of acetic acid, propionic acid, and butyric acid increased over time with no differences among treatments. Feed containing no FMN supported higher concentrations of ammonia and branch chain VFA (BCVFA) versus the other treatments from 6 h through 36 h. There was a high correlation between ammonia and BCVFA concentrations at sample times (r = 0.97). The results indicated that FMN changed ruminal microbial fermentation of protein as measured by changes in BCVFA and ammonia in this *in vitro* fermentation system. The effect of these changes in rumen fermentation on animal performance should be investigated.

Keywords: fumonisin, ruminal bacteria growth, fermentation end products, batch culture.

1. Introduction

Cereal grains produced under drought and severe heat stress are more susceptible to fumonisin contamination [1, 2] which increases the prevalence of fumonisin in feeds fed to dairy cattle. FMN has been shown to cause leukoencephalomalacia in horses [3], pulmonary edema in swine [4], hepatotoxic and nephrotoxic effects in rats [5] and esophageal cancer in humans [6].

It is known that ruminants are less susceptible to FMN than monogastrics. [7] reported growth of pigs was affected when exposed to FMN B1 at 0.1 μg/g. [8] reported that when eighteen steers were fed a diet containing high concentrations of FMN (148 μg/g) for 31 d, feed intake and weight gain were not affected and only two animals had impaired liver function as demonstrated by an increase in the liver enzymes, aspartate aminotransferase, grama glutamyl transpeptidase, and lactatede-hydrogenase. [9] reported that dietary FMN B1 at 100 ppm decreased feed intake and milk production in dairy cattle.

The production data reported are not concordant concerning the extent of ruminal degradation of FMN B1 and its metabolism in the rumen. [10] reported an average degradation of FMN B1 in the buffered system (75:25 of rumen fluid: buffer mix) and 100% rumen fluid of 12.5% and 35%, respectively at the end of a 9 h incubation. The authors found that the method of detection for FMN and the end products and metabolites of breakdown hinge on the presence of the primary amine group. Thus, deamination will directly affect detection. The authors suggested that the observed degradation could be a result of deamination. Since between 65-87% of FMN B1 was intact after 9 h of incubation, the authors suggested that FMN B1 was not easily amenable to hydrolysis by rumen fluid. [11] reported that after 72 h incubation, FMN B1 undergoes only limited (12-18%) biodegradation in the rumen without formation of hydrolysed metabolites and any metabolites, including amino-polyols or aminopentol. The results indicated that FMN B1 was poorly metabolized in the rumen and suggested that such metabolism is not the cause of the tolerance to this toxin displayed by ruminants. [12] studied in vitro ruminal disappearance of FMN B1 and reported that FMN B1 was 10% degraded by ruminal microbes and FMN B1 up to 100 ppm in the diet did not affect in vitro dry matter disappearance rate. However, there was limited data that described the effects of fumonisin on ruminal bacteria growth, and fermentation. The changes in feed intake and animal performance might be related to effects of FMN on ruminal bacterial growth and fermentation.

The objective of this study was to determine the in vitro effects of FMN on microbial growth and fermentation of a lactating dairy cattle diet in batch culture system.

2. Material and Methods

2.1 Incubation System and Experimental Design

Batch culture was used to determine the effects of FMN on ruminal microbial growth and fermentation end products. Rumen fluid was obtained from one lactating fistulated cow fed a total mixed diet formulated to meet nutrient require-ments [13] for a mature cow producing 45 kg/d (Table 1). Rumen fluid was strained through four layers of cheese cloth and diluted 1:3 with McDougall's buffer [14]. Then 120 ml of rumen fluid and buffer mixture was used to inoculate each culture flask containing 3 g of lactating cow diet that had been previously ground to 1 mm. Carbon dioxide was admitted to the head space of the flasks to established anaerobic conditions. Culture flasks were then closed with one way rubber stoppers and incubated

in a continuously shaking water bath at 39°C.

The experiment was arranged as a CRD with six replicates. Eighteen flasks were dosed with lactating TMR containing FMN at 0, 100 and 200 ppm and incubated in a continuously shaking water bath. The levels of FMN used were based on the levels reported to adversely affect milk production and feed intake of dairy cattle by [9] who established FMN toxicity in dairy cattle fed diets containing 100 ppm FMN. In that study, cow were fed FMN at 100 ppm for approximately 7 days prior to calving, through 70 days of lactation, and demonstrated lower milk production (a decrease of 6 kg/cow/day). The 100 ppm FMN also dramatically reduced dry matter consumption and increased serum enzyme concentrations, which are indicative of liver disease. Since [12] reported FMN B1 in the diet up to 100 ppm did not affect in vitro dry matter disappearance rate, this implies that 100 ppm FMN did not affect rumen fermentation. Therefore, this study also included a level two fold higher to determine FMN effects on rumen fermentation.

2.2 Sampling Procedures and Analyses

A 22-gauge needle attached to a 20-ml syringe was used to collect samples through the stoppers of the flasks at 0, 6, 12, 24, and 36 hours of incubation. Each flask was gently swirled before the collection to ensure a representative sample of mixed microbial population and fermentation end products were collected. Samples were placed in collecting tubes and immediately placed in ice to stop microbial growth. Optical density (OD) was measured at 600 nm (Spectrophotometer 20, Bausch & Lomb, USA) and was used as an index of the size of the bacterial population. A representative 5 ml sample was centrifuged at 3000 x g and 4°C for 5 min. Then, 1 ml aliquots of the supernatants were diluted 1:3 with McDougall buffer. The remaining sample was preserved with 50 µl of 6 N HCl/ml [15] and stored frozen (-20°C) until analyzed for VFA and ammonia N. An additional sample was collected and culture pH was measured immediately using an electrode pH meter (Digi-Sense; Cole-Palmer Instrument Co., Chicago, IL/ Model 107, Corning Glass Works, Corning NY).

Table 1. Ingredient and chemical composition of lactating cow diet.

Item	% of Dry matter
Alfalfa hay	15.19
Corn silage	15.96
Alfalfa silage	7.26
Wet brewers grains	7.77
High moisture corn	6.22
Dry corn	19.77
Whole cotton seed	10.33
Soybean hull	5.49
Soybean meal 48%	4.66
Soy Plus®	3.89
Premix [a]	3.46
Nutrient Analysis	**% of DM**
DM	57.41
OM	93.49
CP	17.69
NDF	31.44
ADF	22.08

[a] Premix 1kg: ground corn 45.5%, dicalcium 15%, dynamite 10%, biotin 10%, iodine salt 10%, limestone 3%, Vit A100,000 iu, Vit D 20,000 iu, Vit E 330 iu, Cu 300 mg, Mn 1,500 mg, Zn 1,500 mg, Co 7.5 mg, Se 22.5 mg.

Samples for VFA and ammonia analysis were thawed in the refrigerator and centrifuged at 10,000 x g for 10 min at room temperature. Samples were prepared for VFA as previously described by [16] and analyzed using gas chromatography (Va-

rian; Model 3400; Walnut Creek, CA). Concentration of ammonia was measured according to the procedure of [17] using a DU-65 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

2.3 Statistical Analyses

Data was analyzed as a completely randomized design using PROC MIXED [18] for repeated measures and a comparison of the least square means [19]. Each flask was an experimental unit with treatment, sample time, and interaction between treatment and sample time included as sources of variation in the model. Differences among means of interaction between treatment and sample time were assessed when the F test was significant ($p<0.05$). Significance was declared at $p<0.05$.

3. Results

The effect of FMN on bacteria population size, culture pH and ammonia concentration were reported in Table 2. As measured by changes in OD, the mean size of the bacteria population increased rapidly over time until 12 h of incubation, and culture OD was significantly higher ($p<0.05$) in fermentors fed feed containing FMN at 100 (1.66) and 200 ppm (1.62), versus feed with no FMN (1.41) at 12 h of incubation. As fermentation proceeded, bacteria population sizes significantly decreased ($p<0.05$) at 24 and 36 h, when compared to 12 h within treatments for feed containing FMN at 100 and 200 ppm (1.47 and 1.43 at 24h; 1.34 and 1.32 at 36 h, respectively). However, fermentors fed diet containing no FMN did not decrease (1.33 for 24h and 1.38 for 36 h). After 12 h, the population sizes were not different ($p>0.05$) due to treatments for the remainder of incubation.

Table 2. The effects of fumonisin on bacteria population size (optical density, OD), culture pH, and ammonia concentration over time in batch culture.[1]

Time[2]	Fumonisin (ppm)[3]		
	0	100	200
Optical density			
0	0.51	0.49	0.49
6	0.98	1.13	1.11
12	1.41[b]	1.66[a]	1.62[a]
24	1.33	1.47	1.43
36	1.38	1.34	1.32
Culture pH			
0	7.18	7.29	7.30
6	6.92	6.97	7.00
12	6.61	6.70	6.73
24	6.42	6.56	6.58
36	6.29	6.37	6.40
Ammonia concentration (mM)			
0	0.39	0.40	0.43
6	2.42	1.29	1.15
12	11.94[a]	5.51[b]	5.30[b]
24	23.75[a]	16.27[b]	16.50[b]
6	28.55[a]	21.45[b]	21.50[b]

[1] Standard error of differences of least squares means (SE) = 0.08, 0.03, and 0.89 for OD, culture pH, and ammonia concentration, respectively.
[2] Hours post incubation.
[3] Concentration of fumonisin in diets.
[a, b] Superscripts with different letters within row differed ($p<0.05$).

FMN had no effect ($p>0.05$) on culture pH. Culture pH decreased rapidly from 0 to 6 h of incubation but was similar for all treatments throughout the incubation. At 6 h, culture containing no FMN, 100 ppm FMN and 200 ppm FMN feed had pH of 6.92, 6.97, and 7.00, respectively. Cultures containing no FMN, 100 ppm FMN, and 200 ppm FMN feed had pH at 6.29, 6.37, and 6.40, respectively at the end of the incubation.

After the incubations were started, ammonia concentration was numerically

higher for cultures receiving no FMN (2.42 mM) than 100 ppm FMN (1.29 mM) and 200 ppm FMN (1.15 mM) in feed at 6 h. Concentrations of ammonia were significantly greater ($p<0.05$) in control cultures (11.94 mM) than cultures with feed containing FMN at 100 ppm (5.51 mM) and 200 ppm (5.30 mM) at 12 h until the end of the incubation (28.55, 21.45 and 21.50 mM for culture containing no FMN, 100 ppm, and 200 ppm FMN feed, respectively).

Total VFA, acetic acid, propionic acid, butyric acid concentrations and acetic acid to propionic acid (A:P) ratio are reported in Table 3. Fumonisin had no effect ($p>0.05$) on total concentration of VFA throughout 36 h of the incubation. Total concentrations of VFA increased rapidly from 0 to 6 h and increased over time until the end of the incubation. At the end of the incubation, culture containing no FMN, 100, and 200 ppm FMN in feed had total concentrations of VFA at 108.56, 107.55 and 108.05 mM, respectively.

Acetic acid concentrations increased over time rapidly increasing from 0 to 6 h with no difference due to treatments ($p>0.05$) from the beginning of to the conclusion of the incubation. At the end of the incubation, culture containing no FMN, 100, and 200 ppm FMN in feed had acetic concentrations at 63.51, 61.62 and 62.42 mM, respectively.

Similar to acetic acid concentrations, propionic acid concentrations increased over time with no difference among treatments ($p>0.05$) from the start of the incubation until the end of the incubation. At the end of the incubation, propionic acid concentrations of culture containing no FMN, 100, and 200 ppm FMN in feed were 24.59, 26.38 and 27.32 mM, respectively.

Table 3. The effects of fumonisin on total VFA, acetic acid, propionic acid, and butyric acid concentrations and acetic acid to propionic acid (A:P) ratio over time in batch culture.[1]

Time[2]	Fumonisin (ppm)[3]		
	0	100	200
Total VFA (mM)			
0	27.72	29.29	29.62
6	73.77	66.26	67.84
12	89.91	87.67	89.81
24	106.14	105.05	105.75
36	108.56	107.55	108.05
Acetic acid (mM)			
0	19.03	19.77	19.89
6	46.32	40.50	41.26
12	54.40	51.10	52.66
24	62.56	63.11	61.30
36	63.51	61.62	62.42
Propionic acid (mM)			
0	5.51	5.91	5.98
6	16.81	16.91	17.30
12	20.84	22.51	23.51
24	24.85	26.23	27.05
36	24.59	26.38	27.32
Butyric acid (mM)			
0	2.58	2.73	2.75
6	8.19	7.08	7.25
12	11.35	10.59	10.73
24	13.93	12.99	13.01
36	14.42	13.49	13.14
A:P ratio			
0	3.34	3.34	3.32
6	2.72^{a}	2.41^{b}	2.39^{b}
12	2.59^{a}	2.21^{b}	2.25^{b}
24	2.49^{a}	2.26^{b}	2.27^{b}
36	2.56^{a}	2.26^{b}	2.29^{b}

[1] Standard error of differences of least squares means (SE) = 2.97, 2.10, 0.76, 0.42, and 0.02 for total VFA, acetic acid, propionic acid, and butyric acid concentra-tions, and acetic acid to propionic acid ratio, respectively.

[2] Hours post incubation.

[3] Concentration of fumonisin in diets.

[a, b] Superscripts with different letters within row differed ($p<0.05$).

Butyric acid concentrations increased rapidly from 0 to 6 h, with similar change over time comparing to other VFA concentrations with no differences among treatments ($p>0.05$). At 36 h, cultures containing no FMN, 100, and 200 ppm FMN in feed had butyric acid concentration of 14.42, 13.49, and 13.14 mM, respectively.

Acetic acid to propionic acid ratio was significantly lower in cultures containing 100 and 200 ppm FMN in feed versus the culture containing no FMN in feed from 6 to 36 h. At 36 h, acetic to propionic acid ratios in cultures containing no FMN, 100 ppm, and 200 ppm FMN were 2.56, 2.26 and 2.29, respectively.

Branch chain VFA (BCVFA) concentrations which included isobutyric acid, valeric acid, isovaleric acid concentrations were reported in Table 4. Isobutyric acid, valeric acid and isovaleric acid concentrations increased over time. Concentrations of all BCVFA increased steadily throughout the 36 h incubation and were significantly lower ($p<0.05$) for culture with feed containing FMN at 100 and 200 ppm than feed containing no FMN. There were significantly lower ($p<0.05$) concentrations of BCVFA in cultures containing 100 and 200 ppm FMN feed compared to the culture with no FMN. These differences were measured from 6 h to the end of the incubation. At the end of the incubation, BCVFA concentrations in cultures containing no FMN, 100 ppm and 200 ppm FMN in feed were 6.48, 5.22, and 5.07 mM, respectively.

As reported in Figure 1, there was a high correlation ($p<0.0001$) between ammonia and BCVFA concentrations for all treatments incubated for 36 h in this incubation system ($r = 0.97$).

Table 4. The effect of fumonisin on isobutyric acid, valeric acid, isovaleric acid, and branch chain VFA concentrations over time in batch culture.[1]

Time[2]	Fumonisin (ppm)[3]		
	0	100	200
Isobutyric acid (mM)			
0	0.18	0.18	0.20
6	0.49[a]	0.33[b]	0.38[b]
12	0.71[a]	0.51[b]	0.49[b]
24	1.01[a]	0.81[b]	0.81[b]
36	1.39[a]	1.01[b]	1.01[b]
Valeric acid (mM)			
0	0.34	0.39	0.38
6	1.27[a]	1.01[b]	1.01[b]
12	1.56[a]	1.43[b]	1.46[ab]
24	2.09[a]	1.92[b]	1.98[ab]
36	2.63[a]	2.29[b]	2.18[b]
Isovaleric acid (mM)			
0	0.29	0.31	0.33
6	0.92[a]	0.57[b]	0.53[b]
12	1.26[a]	0.88[b]	0.81[ab]
24	2.03[a]	1.48[b]	1.49[ab]
36	2.47[a]	1.91[b]	1.88[b]
Branch chain VFA (mM)[4]			
0	0.79	0.88	0.91
6	2.67[a]	1.90[b]	1.90[b]
12	3.53[a]	2.81[b]	2.76[b]
24	5.12[a]	4.21[b]	4.29[b]
36	6.48[a]	5.22[b]	5.07

[1] Standard error of differences of least squares means (SE) = 0.05, 0.08, 0.05 and 0.16 for isobutyric acid, valeric acid, isovaleric acid and branch chain VFA concentrations, respectively.

[2] Hours post incubation.

[3] Concentration of fumonisin in diets.

[a, b] Superscripts with different letters within row differed ($p<0.05$).

[4] Branch chain VFA concentration includes the concentrations of isobutyric acid, valeric acid and isovaleric acid.

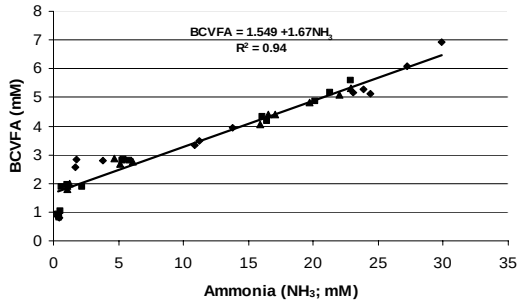

Figure 1. Correlation between ammonia and branch chain volatile fatty acid (BCVFA) concentrations of diets containing fumonisin at 0 (♦), 100 (■) and 200 (▲) ppm incubated in batch culture (r = 0.97; P<0.0001).

4. Discussion

Bacterial population size was lower in the control culture versus cultures containing FMN at 12 h. However, bacterial population size was not different due to treatment after 12 h of incubation possibly due to bacterial adjustment to FMN.

Ammonia concentrations range 0.39 to 0.43 mM and were low at the beginning of the incubation. However, the concentrations were higher than the limiting concentration for adequate microbial growth (0.1 mM) suggested by [20]. The level of ammonia in the current study was adequate as illustrated by the rapid growth of bacteria in all three treatments after the incubation was started.

There was a decrease in the concentration of ammonia in the batch cultures containing FMN at either 100 or 200 ppm, suggesting proteolytic bacteria were inhibited by FMN causing a decrease in protein degradation. [21] reported that reducing-equivalent disposal is a major effector of the metabolism of BCVFA in bacteria. A high correlation (r = 0.97; P<0.0001) between ammonia and BCVFA concentrations existed at 36 h of incubation. Fumonisin may possibly inhibit protein fermentation through a disruption of redox status via the NADH to NAD ratio. This result is in contrast with [11]. [11] concluded that FMN at 1 μg/ml did not affect activity of ruminal microflora. It is important to note that those investigators did not report all BCVFA or ammonia concentrations.

Total VFA concentration in the batch culture system was not changed by the presence of FMN. Acetic acid, propionic acid and butyric acid concentrations were also not different among treatments throughout the sample times reflecting no effect of FMN on carbohydrate fermentation. These results agree with the data of [11] who reported FMN at 1 μg/ml did not affect acetic, propionic, or butyric acid concentrations during 72 h of incubation. Our study used higher concentration of FMN (up to 5 μg/ml) illustrating that at even higher levels of FMN, production of the major VFAs was not altered. While total VFA and major VFAs concentration were not changed, the concentration of branch-chained VFAs (BCVFA) was significantly decreased by the addition of FMN to the batch cultures.

Although there was no effect of FMN on carbohydrate fermentation, as evidenced by no change in total VFA or the major VFAs, feed with FMN at 100 and 200 ppm exhibited significantly lower A:P ratio than the control throughout the experiment. This difference suggests there was a shift of rumen bacteria with a decrease in cellulolytic bacteria and an increase in starch digesting bacteria that might decrease fiber digestion [22] due to FMN. This result in the current study contrasts with [12] who reported that up to 100 ppm FMN B1 in the diet did not affect in vitro dry matter disappearance rate.

Culture pH throughout the incubation was not different among treatments in the present study. Given that no differences were present among treatments in total concentration of VFA, these results agree

with known relationships between VFA production and pH.

5. Conclusion

Fumonisin increased bacterial growth during the first 12 hours of batch culture. The inclusion of FMN up to 200 ppm in feed (5 μg/ml) did not affect the production of the major VFAs. Fumonisin did decrease BCVFA concentration with a concurrent decrease in ammonia concentration. The results from this experiment indicate that there is a shift in rumen bacteria population and change in ruminal microbial fermentation due to the presence of FMN in the feed.

6. Implications

Decreased protein degradation with the disruption of redox status, via the NADH to NAD ratio associate with FMN needs to be investigated. Additional research is necessary to determine the effect of these responses on dietary and microbial flow in vitro and in vivo.

7. References

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