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Effects of patulin on rumen microbial fermentation in continuous culture fermenters

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Abstract

Eight single-flow continuous culture fermenters were used to study the effects of different concentrations of patulin on rumen microbial fermentation. Two 1 l fermenters were spiked with 0, 30, 60 or 90 mg of patulin every 12 h for 3 consecutive days. True digestion of organic matter (TOM), acid detergent fiber (ADF) and crude protein (CP) decreased ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) with patulin addition ranging from 30 to 90 mg. Bacterial nitrogen flow was lower ($P < 0.01$) with patulin addition (30–90 mg) compared with the control treatment, whereas, the efficiency of bacterial growth (g of N/kg OMTD) was lowest ($P < 0.05$) when 90 mg of patulin were added to fermenter flasks. Total volatile fatty acid (VFA) concentration (mM) in fermenter effluents decreased ($P < 0.05$) from 180.1 to 119.5 with the addition of 90 mg of patulin but did not differ ($P > 0.05$) between the control treatment and 30–60 mg of patulin. Acetate (mol/100 mol) was depressed ($P < 0.01$) with patulin addition. Conversely, there was an increase ($P < 0.05$) in the molar proportion of butyrate and valerate in the fermenters treated with the toxin. At the highest level of patulin addition (60 and 90 mg), branched-chain VFA were lower ($P < 0.01$) probably due to a reduction in protein (branched-chain amino acid) degradation. It is concluded that patulin can alter metabolism of nutrients by ruminal microbes. These changes could potentially have a negative impact on animal health and performance.

Keywords: Mycotoxins; Patulin; Ruminal fermentation; Continuous culture

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1. Introduction

Patulin, 4-hydroxy-4H furo (3,2C) pyran-2 (6HO)-one, is a secondary metabolite of toxigenic strains of *Penicillium*, *Aspergillus* and *Byssochlamys* species (Palmgren and Ciegler, 1983). These molds are common contaminants of fermented feeds. Patulin is toxic to a wide range of organisms including microbes, plants, and animals (for complete review see Singh, 1967; Stott and Bullerman, 1975; McKinley and Carlton, 1991). It has an antimicrobial effect on aerobic gram positive and gram negative bacteria (Singh, 1967) and also affects anaerobic bacteria (De Rosnay et al., 1952, cited by Singh, 1967).

Reports of the effects of patulin on cattle are rare but there is some evidence that patulin can have a negative impact on ruminants. Patulin in silage has been associated with hemorrhagic disorders in English cattle (Syret, 1977). Other patulin-related diseases have been reported in Japan, France, and Germany when cattle were fed moldy fermented feeds (Hori et al., 1954; Moreau and Moreau, 1960; Jacquet et al., 1963; Schultz et al., 1968).

There are two studies on the effect of patulin on microbial fermentation in the rumen. In one study, microbial protein and acetic acid production were reduced in batch cultures exposed to 100 μg patulin/ml of culture solution (Escuola, 1992). In the second study, in vitro dry matter (DM) and organic matter (OM) digestibilities of wheat straw were reduced when 10 nmol of patulin were added to the culture media (Abdelhamid et al., 1992). The objective of this study was to describe the effects of different concentrations of patulin on rumen microbial fermentation in continuous culture fermenters.

2. Materials and methods

2.1. Patulin source

Patulin was produced by growing *Penicillium griseofulvum* NRRL 5256 on potato dextrose broth (Difco) at 28 °C for 4 weeks. The mycelia and broth were then extracted twice with ethyl acetate. Extracts were pooled, evaporated to dryness in a rotary evaporator, then reconstituted in chloroform. Extracts were kept at −18 °C until they were used.

Immediately prior to use, aliquots of the chloroform extract were evaporated to dryness under a N_2 stream, reconstituted in methanol, filtered through a 0.45 μm filter, then patulin was quantitated by HPLC. A Rabbit HP (Rainin Instrument Co., Woburn, MA) HPLC pump, a Spectroflow 757 UV absorbance detector, and a 3392[a] integrator (Hewlett-Packard, Palo Alto, CA) were used. The column was a 150 mm × 4.60 mm reversed phase LUNA 5 mm C_8 (Phenomenex, Torrance, CA). The mobile phase was water:acetonitrile (95:5) at a flow rate of 1 ml/min. Patulin was detected by UV absorbance at 276 nm. Chloroform extracts were evaporated to dryness in rotary evaporator, then the dry residue was exposed to N_2 air stream for 1 h to achieve complete elimination of chloroform. Dry residue was then reconstituted in distilled water immediately before addition to the continuous culture fermenters.

2.2. Continuous culture system and operation

The experiment was conducted in two 7-day periods, with 4 days for stabilization and 3 days for patulin addition and sample collection. Eight single-flow continuous culture

fermenters (Hannah et al., 1986) were inoculated with ruminal fluid from one canulated cow fed a 70:30 forage:concentrate diet. Rumen fluid was collected after the morning feeding, strained through two layers of cheesecloth and kept in a thermos until inoculation into the fermenter flasks.

Fermenters were provided with 75 g of DM per day of a pelleted diet in eight equal parts by an automated feeding device. The pelleted diet contained 38% alfalfa hay, 28% corn silage, 27% cracked corn, 5% soybean meal, and 0.6% mineral mix (DM basis). The chemical composition of the pelleted diet was: 92.9% organic matter (OM), 15.5% crude protein (CP), 27.4% NDF, 16.2% ADF (DM basis).

Two fermenters were spiked with 0, 30, 60 or 90 mg of patulin in 1 ml of water every 12 h for the last three consecutive days of each period. Flow rate of each fermenter was set at 6% per h by regulating the buffer input. The culture pH was recorded every 10 min by an electronic data acquisition system (Daisy Lab) and was maintained between 6.0 and 6.5 by automated addition of either 5N NaOH or 3N HCl. Anaerobic conditions were maintained by continuous infusion of N_2. The fermenter temperature was maintained at 38.5 °C.

2.3. Sample collection and analytical procedures

During the sampling period, fermenter effluents were maintained at 2 °C in a water bath to retard microbial and enzymatic activities. Fermenter effluents were homogenized, and three separate 500 ml aliquots were removed daily and composited by fermenter. Composite effluent samples were analyzed for total N, NH_3-N, and volatile fatty acids (VFA).

Freeze-dried composite samples were analyzed for DM, OM, fiber, ash, and purines. At the end of each experimental period, the contents from each fermenter were strained through two layers of cheesecloth, centrifuged at $1000 \times g$ for 10 min to remove feed particles, then the supernatant was centrifuged at $20{,}000 \times g$ for 20 min to separate bacteria. Bacterial pellets were resuspended in distilled water, frozen and lyophilized. Purine concentrations were determined by the method of Zinn and Owens (1986). Purine contents of effluents and bacteria were used to partition effluent N flow into microbial and dietary nitrogen. Total N in the effluent, bacteria, and diet was determined by the Kjeldahl method (AOAC, 1984). Ammonia N was determined by steam distillation using a Kjeltech 2300 Analyzer Unit (Tecator, Herdon, VA). Effluent VFA concentrations were measured by gas chromatography (Hewlett-Packard, model 5880A, Palo Alto, CA) with a Carbopack DA/0.3% Carbowax 20M column (Supelco, Bellefonte, PA). Sequential detergent fiber analyses (Van Soest et al., 1991) were used to determine NDF and ADF concentrations of the diet and effluents.

2.4. Statistical analysis

Data were analyzed as a randomized complete block design using the GLM procedure of SAS (1989). The linear model used for each dependent variable was: $Y_{ij} = \mu + P_i + T_j + \varepsilon_{ij}$, where μ is the common mean; P_i, the period (block); T_j, the treatment; and ε_{ij}, the random error. The LSD test was used for mean comparison among treatments.

Table 1
Effects of different concentrations (mg/l) of patulin on pH and digestion in continuous culture fermenters

Item	Patulin concentration (mg/l)				MSE	*P*-value
	0	30	60	90		
pH	6.21	6.22	6.26	6.32	0.02	0.2
Digestion (%)						
Apparent OM	26.1	22.9	18.7	24.4	6.2	0.50
True OM[a]	43.0 a	32.9 b	29.0 b	32.3 b	4.1	0.02
ADF	28.7 a	15.7 b	16.9 b	16.5 b	5.1	0.005

Means within a row with diffferent letters differ.
[a] Corrected for contribution of bacterial OM in the effluent.

3. Results and discussion

Patulin adversely affected digestion, nitrogen metabolism and energy utilization in a dose-dependent manner.

3.1. Digestion

Apparent OM digestion was similar among treatments. However, true OM digestion, corrected for bacterial matter, decreased ($P < 0.05$) with the addition of patulin (Table 1). These results are in agreement with the observations from Abdelhamid et al. (1992) who reported that 10 nmol of patulin produced a significant reduction of the in vitro DM and OM digestion of wheat straw.

Similarly, digestion of ADF also decreased ($P < 0.01$) by addition of patulin (Table 1). Decreases in true OM and ADF digestion were correlated with a decrease in the bacterial N content of the effluents ($r = 0.69$ and 0.55, respectively) (Table 2).

3.2. Nitrogen metabolism

The effect of patulin on rumen nitrogen metabolism is shown in Table 2. Nitrogen intake was the same in each fermenter. Differences in total and non-ammonia N flows and in bacterial N as a percentage of fermenter DM were not detected between control and treatment fermenters.

CP digestion and bacterial N flow were reduced by patulin treatment ($P < 0.05$ and $P < 0.01$, respectively). The decrease in bacterial N flow correlated with the greater ($P < 0.05$) concentration of dietary N outflow observed in patulin-treated fermenters.

The control fermenters had NH_3-N concentrations of 5.96 mg/dl. This concentration exceeded the 5 mg/dl suggested by Satter and Slyter (1974) for optimum microbial growth. Bacterial N outflow from control fermenters in the current study was 1.13 g per day, similar to that recently reported in previous studies (Bach et al., 1999).

Ammonia N concentration in the fermenters spiked with 30 and 60 mg of patulin was reduced ($P < 0.05$) with respect to the 90 mg treatment, but not with respect to the

Table 2
Effects of different concentrations of patulin (mg/l) on ruminal nitrogen metabolism in continuous culture fermenters

Item	Patulin concentration (mg/l)				MSE	*P*-value
	0	30	60	90		
N intake (g per day)	2.51	2.50	2.49	2.50	0.01	0.27
N flow (g per day)						
Total	2.02	1.98	2.03	1.93	0.17	0.90
Ammonia	0.10 a,b	0.06 b	0.06 b	0.16 a	0.04	0.04
Non-ammonia	1.92	1.92	1.96	1.77	0.20	0.60
Bacterial	1.13 a	0.65 b	0.65 b	0.44 b	0.10	0.0004
Dietary	0.79 a	1.27 b	1.32 b	1.32 b	0.19	0.03
NH_3-N concentration (mg/dl)	5.96 a	4.03 a	4.32 a	10.15 b	2.40	0.02
CP degradation (%)	64.5 a	42.9 b	40.9 b	40.5 b	8.8	0.02
Bacterial N (% of DM)	8.03	6.83	7.35	6.27	1.35	0.45
EMPS	39.0 a	28.3 a,b	31.9 a,b	19.7 b	7.5	0.04
ENU	0.66	0.53	0.55	0.39	0.10	0.07

EMPS: efficiency of microbial protein synthesis (g of bacterial N/kg of OM truly fermented in continuous culture). ENU: efficiency of N utilization by ruminal bacteria [(g of microbial nitrogen/g of ruminal available nitrogen) × 100]. Means within a row with different letters differ.

controls. The NH_3-N concentration in fermenter fluid depends on the extent of protein degradation and rate of N uptake by the microbes. In the present experiment, CP degradation and bacterial N flow were reduced in all patulin-treated fermenters. An equivalent decrease in both production and utilization of ammonia may explain the absence of a change in NH_3-N concentration between the 30 and 60 mg/l fermenters. Ammonia N concentrations in these fermenters were below 5 mg/dl. This possible limitation in available NH_3-N may be partially responsible for the observed low bacterial N flow.

The NH_3-N concentration increased ($P < 0.05$) in the 90 mg treatment. In vitro rumen fermentation studies using long term rumen simulation (RUSITEC), demonstrated a 68 and 35% increase in NH_3-N concentrations for moldy corn silage and moldy grass, respectively (Maiworm et al., 1995; Holtershinken et al., 1997). The main cellulolytic bacterial species utilize ammonia as their main source of N for microbial protein synthesis. A decrease in this bacterial population or in its efficiency ($P = 0.07$) of N utilization (g of microbial nitrogen/g of ruminal available nitrogen) could lead to an accumulation of NH_3-N in the effluents. Escuola (1992) reported an inhibition of bacterial protein synthesis when patulin was added to rumen microflora under batch culture conditions. It is also possible that the ability of the ruminal bacteria to degrade the urea provided by the saliva might have exceeded the ability of bacteria to capture the resulting ammonia into bacterial protein.

The efficiency of bacterial synthesis expressed as grams of bacterial N per kg of OM truly digested, was affected ($P < 0.05$) by patulin treatment. Values ranged from 39.0 in fermenters without patulin to 19.7 g of N/kg of OM truly digested in fermenters treated with the highest concentration of toxin. The efficiency of bacterial synthesis depends on many dietary and ruminal factors. Whether this decrease was due to a direct or indirect antimicrobial effect of patulin is discussed in the VFA section below.

Table 3
Effects of different concentrations (mg/l) of patulin on VFA concentration in continuous culture fermenters

Item	Patulin concentration (mg/l)				MSE	*P*-value
	0	30	60	90		
Total VFA (mM)	180.1 a	153.5 a,b	159.9 a,b	119.5 b	23.3	0.04
Individual VFA, mol/100 mol						
Acetate	63.6 a	41.5 c	44.7 c	48.8 b	4.3	0.001
Propionate	19.0	25.2	26.4	19.0	4.6	0.08
Butyrate	13.2 a	25.6 b	21.1 a,b	26.4 b	5.0	0.04
Branched-chain VFA	2.1 b	2.9 a	1.0 c	0.6 c	0.4	0.001
Valerate	2.1 b	4.6 a	5.8 a	4.8 a	0.5	0.03
Acetate:propionate	3.4	1.7	1.7	2.8	0.7	0.05

Means within a row with different letters differ.

4. VFAs

Total VFA production (mM) in fermenter effluent was reduced ($P < 0.05$) from 180.1 to 119.5 by 90 mg of patulin but it was not affected by 30 and 60 mg of toxin (Table 3). Molar proportions of acetate decreased ($P < 0.01$) with patulin addition. This was consistent with low digestibilities of OM and ADF (Table 1) and may be the result of a modification of the cellulolytic bacterial population. Conversely, there was an increase ($P < 0.05$) in the molar proportion of butyrate in the fermenters treated with toxin. Molar proportions of propionate were not affected by any level of patulin. Holtershinken et al. (1997) reported a reduction of 9.9 and 12.2% in the production of acetate and propionate, respectively, and an increase of up to 39.9% in *n*-butyrate in continuous culture of cow ruminal fluid fed with moldy grass.

The highest additions of patulin (60 and 90 mg) induced a reduction in branched-chain fatty acid (BCVFA) production ($P < 0.01$). Branched-chain amino acids are precursors to some BCVFA. Some BCVFA are essential growth factors for certain ruminal bacteria, particularly the cellulolytic species (Bryant, 1973). Low CP degradation (Table 2) may have led to low BCVFA concentrations and subsequent reductions in cellulolytic activity.

Valerate molar proportions increased in the patulin-treated fermenters ($P < 0.05$). Valerate is one of the minor fermentation end-products of gram negative bacteria (Stewart and Bryant, 1988). Increases in its molar proportions have been associated with a shift in rumen microbes towards gram negative bacteria after addition of antimicrobial agents (Barry et al., 1978; Sauer and Teather, 1987).

5. Conclusions

Patulin concentrations of 30, 60 and 90 mg adversely affected ruminal microorganism function and production of bacterial end-products in continuous culture fermenters. Assuming a rumen volume of about 150 l and consumption of 30 kg DM per day, patulin must be present at 150 mg/kg DM of the diet to give a rumen concentration of 30 mg patulin/l. Patulin concentrations of 1.5–40 ppm were reported from corn silage samples

(Escuola, 1974). Pure cultures of molds isolated from these silage samples produced 90–300 ppm of patulin (Escuola, 1974).

The ruminal microbial population is an integrated system with numerous interrelationships and any observed result or modification is likely a combination of several interactions. Therefore, changes in microbial fermentation and production of bacterial protein and bacterial end-products could be attributed to a direct inhibitory effect of patulin on bacterial growth or to a limitation in the availability of certain essential nutrients as result of alterations in the food chain interactions in the rumen ecosystem.

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