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Prevention of Patulin Toxicity on Rumen Microbial Fermentation by SH-Containing Reducing Agents

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Patulin, a toxic fungal metabolite, negatively affects rumen fermentation. This mycotoxin has also been associated with intoxication cases in cattle. This study investigates the use of SH-containing reducing compounds to prevent patulin's negative effects on the rumen microbial ecosystem. The effect of 50 μg/mL patulin on the fermentation of alfalfa hay was measured in batch cultures with and without reducing agents. Sulfhydryl-containing cysteine and glutathione prevented the negative effects of the toxin on dry matter degradation, gas, and volatile fatty acid production ($P < 0.01$). However, non-sulfhydryl-containing ascorbic and ferulic acids did not protect against patulin's toxicity ($P > 0.01$). Patulin was unstable in buffered rumen fluid as the concentration decreased by half after 4 h of incubation. In the presence of sulfhydryl groups, the toxin disappeared rapidly and was not detected after 1 h of incubation. The utilization of sulfhydryl-containing compounds such as cysteine to avert patulin toxicity could have practical implications in ruminant nutrition.

KEYWORDS: Patulin; mycotoxin; *Byssochlamys nivea*; *Penicillium expansun*; rumen fermentation; cysteine

INTRODUCTION

Patulin is a secondary toxic metabolite produced by fungi of the genera *Penicillium*, *Aspergillus*, and *Byssochlamys*. The species most commonly encountered in animal feeds is *B. nivea*, a frequent contaminant in air-exposed, cutting fronts of silages (*1*). *P. granulatum* has also been associated with patulin contamination in silages (*2*). Other feeds susceptible to contamination by patulin are barley malt residues, which can be colonized by patulin-producing strains of *A. clavatus* and *P. urticae*, and cereal stubbles, which can be colonized by *A. clavatus*. Also, apple industry byproducts are often contaminated by *P. expansum*, especially if prepared from decayed fruits. The presence of patulin in apple food products has been the subject of several published surveys (reviewed in ref *3*).

Patulin is an electrophilic molecule that exerts its deleterious effects by binding covalently to cellular sulfhydryl (SH, thiol) groups of proteins and glutathione (*4*, *5*). This affinity to SH groups can be used to detoxify the toxin (*6*). The adducts of patulin and SH-containing compounds are generally less toxic than patulin alone, although they may remain slightly bactericidal and bacteriostatic (*6*, *7*). Other reducing agents, such as ascorbic acid, have also been reported to inactivate patulin (*8*, *9*).

Patulin is a broad-spectrum antibiotic and has been demonstrated to be toxic, teratogenic, carcinogenic, and mutagenic in several bacterial, cellular, and animal systems (*4*, *10*, *11*). In cattle this toxin has been associated with the death of more than 100 cows fed malt (*4*) and to hemorrhagic syndrome and death in silage-fed cattle (*12*). In contrast to the high prevalence of patulin-producing fungi found in silages, there have been relatively few reported cases of toxicosis in ruminants (*1*, *13*). Patulin's toxic effects are unspecific, and certainly most patulin toxicoses remain undiagnosed. Mild intoxications could be manifested only by an increased susceptibility to secondary pathologies and a decrease in production efficiency. Indeed, patulin has a marked negative impact on rumen microbes and feed fermentation in vitro (*14*, *15*).

The objective of this work was to evaluate whether the negative effect of patulin on rumen fermentation could be reversed by the addition of reducing compounds.

MATERIALS AND METHODS

Chemicals and Preparation of Rumen Fluid. Patulin from Sigma-Aldrich was used as a 10 mg/mL solution in deionized sterile water. Glutathione, ferulic acid, L-ascorbic acid sodium salt, and DL-cystine were from Sigma-Aldrich, and L-cysteine HCl was from Fluka. Solutions were freshly prepared in deionized sterile water at 0.2 M concentrations except for cystine, which was prepared at a concentration of 0.1 M and used as a suspension.

Rumen fluid was obtained from three rumen-fistulated wethers fed a hay diet twice daily, at 8:00 a.m. and 4:00 p.m. All animals were cared for in accordance with the guidelines for animal research of the French Ministry of Agriculture. Whole rumen contents were collected before the morning feeding and strained through a double layer of gauze

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Table 1. Effect of Patulin Concentration on in Vitro Rumen Fermentation[a]

	patulin				
	0 μg/mL	25 μg/mL	50 μg/mL	100 μg/mL	SEM
dry matter degradation (%)	44.1 a	38.6 b	33.4 c	32.1 c	1.02
gas production (mL)	8.3 a	5.8 b	4.5 c	3.0 d	0.15
volatile fatty acids total (mmol L^{-1})	70.2 a	62.1 b	49.3 c	36.5 d	1.86
mol per 100 mol					
acetate (A)	65.7 a	59.9 b	60.2 b	62.3 c	0.54
propionate (P)	24.7 a	29.7 b	28.5 c	28.1 c	0.58
butyrate	6.2 a	7.2 b	8.9 c	7.4 b	0.08
AP ratio	2.7 a	2.0 b	2.1 bc	2.2 c	0.09

[a] Within a row, means followed by different letters differ ($P < 0.05$).

Table 2. Prevention of Negative Effects of Patulin on in Vitro Rumen Fermentation by Reducing Agents[a]

	control		gluthathione		cysteine		cystine		ferulic acid		ascorbic acid		
	no Pat	+ Pat	no Pat	+ Pat	no Pat	+ Pat	no Pat	+ Pat	no Pat	+ Pat	no Pat	+ Pat	SEM
dry matter degradation (%)	44.2	26.5**	44.4	43.8	43.6	43.9	42.1	28.1**	42.8	26.5**	43.9	26.1**	0.81
gas production (mL)	8.2	1.9**	8.2	8.1	8.7	8.1	7.5	1.9**	7.6	1.5**	8.3	2.0**	0.19
volatile fatty acids total (mmol L^{-1})	69.9	25.0**	75.5	72.9	71.7	63.9**	67.4	29.1**	69.8	28.3**	75.1	26.3**	1.36

[a] **, $P < 0.01$. Comparisons were made between treatments with and without 50 mg/mL patulin. Reducing agents used at a concentration of 4 mM.

under a stream of O_2-free CO_2 to remove solids. Equal volumes of rumen fluid from each animal were pooled and then mixed in a 1:5 ratio with an anaerobic buffer solution (*16*) kept at 39 °C under O_2-free CO_2 gas. This rumen fluid−buffer mixture was utilized immediately after preparation for fermentation experiments.

In Vitro Rumen Fermentation Experiments. Five milliliters of the rumen fluid−buffer mixture was transferred to Hungate tubes containing 100 ± 2 mg of alfalfa hay followed by the addition of a solution of patulin, reducing agents, or both. The tubes were capped to maintain the anaerobic conditions in the headspace and incubated horizontally at 39 °C in a shaking water bath for 18 h. At the end of the incubation period, gas production was monitored by piercing the stoppers with a needle connected to a buret filled with water and measuring water displacement. Contents were then immediately filtered through preweighed sintered glass crucibles (porosity 1, 100−160 μm pore size). Residues were dried at 60 °C for 48 h to determine dry matter degradation (DMD) and then ashed at 550 °C for 8 h to determine organic matter degradation (OMD). The filtrates were used to measure pH after incubation. For volatile fatty acids (VFA) determination 2 mL of filtrate was mixed with 0.2 mL of 5% (v/v) metaphosphoric acid and stored at − 20 °C until analysis. The first series of experiments was performed for two purposes: (1) to validate the negative effect of patulin on rumen microbial fermentation in this minifermentation system and (2) to select an appropriate concentration for testing the effects of the reducing agents. Patulin concentrations of 0, 25, 50, and 100 μg/mL were used for this purpose. For experiments testing the protective effect of different reducing agents, the intermediate concentration of 50 μg/mL (∼0.3 mM) patulin in the final mixture was selected as this concentration induced changes in fermentation characteristics that would be easily detectable. Reducing agents were tested at concentrations of 4 mM except for cystine, which was used at a concentration of 2 mM. The protective action of cysteine was further assayed using increasing concentrations of this amino acid. All treatments were assayed in triplicate and repeated in time.

In another experiment, changes in patulin concentration in the presence or absence of 0.2 and 0.4 mM cysteine were followed throughout the incubation time. At the same time the concentration of free sulfhydryl groups in the fermentation media was monitored. Flasks containing 200 ± 2 mg of alfalfa hay and 20 mL of rumen fluid−buffer mixture were incubated in triplicate as described above. Samples (2 mL) were taken at different times during the incubation period and processed immediately. For patulin determination, a 1 mL sample was mixed with 3 mL of ethyl acetate, mixed using a rotator set at 60 rpm for 15 min, and centrifuged, and 2 mL of supernatant was recovered. Supernatants were evaporated to dryness at 40 °C under a stream of nitrogen gas, the residue dissolved in 200 μL of methanol-distilled water (1:4, v/v) and used for analysis. For free sulfhydryl group determination, the rest of the sampling volume was centrifuged at 15000*g* for 10 min at room temperature and the supernatant used for analysis.

Analysis. VFA were analyzed by gas chromatography using a wall-coated open-tubular fused silica column (0.25 mm i.d. × 25 m) coated with CP-Wax 58 (FFAP)-CB. Nitrogen was used as carrier gas with a split system. The temperature of the column was set at 130 °C for 1 min, increased at a rate of 15 °C/min to 220 °C, and then held for 2 min. The injector and flame ionization detector were set at 220 and 250 °C, respectively. Patulin was analyzed by HPLC using a Nucleosil C18 column and UV detection (275 nm). Sulfhydryl groups in the fermentation were detected with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) following the method of Ellman (*17*). Data were statistically analyzed by one-way analysis of variance using the General Linear Models procedure of SAS (SAS Institute Inc., Cary, NC). Significance was declared at the 5% probability level, and differences among means were tested using the Tukey option (Tukey−Kramer method). Linear contrasts were performed using an orthogonal design with coefficients selected to account for unequally spaced treatments.

RESULTS

The addition of patulin to the rumen fluid mixture negatively affected ($P < 0.05$) fermentation in vitro (**Table 1**). Increasing concentrations of patulin in the incubation media had a negative linear effect ($P < 0.01$) on all fermentation variables measured. The highest concentration of patulin used (100 μg/mL) decreased DMD by 27%, whereas gas and VFA production decreased by 64 and 48%, respectively. Even at the low concentration of 25 μg/mL the toxin decreased ($P < 0.05$) DMD and VFA by 12% and gas production by 30%. Patulin caused a shift in the acetate/propionate ratio from 2.7 in control to 2.0 with maximal effect at the lowest concentration (25 μg/mL). OMD had the same tendency as DMD results for this and subsequent experiments, and data are not shown.

Table 2 shows the effects of reducing agents used to prevent patulin's toxicity. In the absence of patulin, none of the reducing agents negatively affected the fermentation variables studied ($P > 0.05$). In contrast, addition of glutathione and ascorbic acid tended to increase production of VFA. In the presence of patulin only those compounds containing an active thiol group, for example, cysteine and glutathione, were effective in blocking

the toxin's effects. Glutathione completely inhibited the negative effect of patulin. Cysteine appeared to be slightly less effective than glutathione as VFA production did not equal that of controls. However, in the second experiment (shown below) the same cysteine concentration of 4 mM effectively prevented patulin's negative effects on all of the fermentation variables measured. In contrast, cystine, a dimeric amino acid readily found in nature that contains two molecules of cysteine linked via a disulfide bridge, did not have any positive effect. Apparently, the disulfide bridge in cystine prevents utilization of the thiol group as a reducing functionality as compared to cysteine. Other common reducing agents present in feeds, such as ascorbic and ferulic acid, did not prevent the harmful effect of the toxin on the fermentations.

Cysteine, a nonessential amino acid, is a natural component of feed protein supplements. This amino acid can also be added to ruminant's diets as an additive. Because of the potential practical utilization of cysteine, the lowest dose able to prevent the toxic effect of 50 μg/mL (~0.3 mM) patulin was tested. Increasing concentrations of cysteine prevented patulin effects proportionally (**Figure 1**). The linear effect of the cysteine dose was highly significant ($P < 0.001$) for all variables measured. A 1 mM concentration cysteine completely neutralized the harmful effects of patulin on fermentation. Cysteine concentrations of 0.2 and 0.4 mM decreased the negative effect of patulin on fermentation by as much as 50 and 75% ($P < 0.01$), respectively.

Although highly toxic to the in vitro rumen fermentation, patulin was not stable in rumen contents. The concentration of toxin when incubated alone decreased markedly throughout the incubation. After 4 h, it decreased by 50%, and it was almost not detected at the end of the 18-h incubation period (**Figure 2A**). In the presence of cysteine, patulin decreased dramatically from the start of the incubation, indicating the formation of cysteine−toxin adducts. Patulin concentration decreased below the lower limit of detection of 1 ng/mL after 2 and 6 h of incubation in the presence of 0.4 or 0.2 M cysteine, respectively. Concomitantly to patulin measurements, the presence of free SH groups was monitored. In treatments containing both patulin and cysteine it was observed that the disappearance of patulin corresponded with the loss of SH groups from the system (**Figure 2B**). In treatments containing 0.4 and 0.2 M cysteine alone SH groups at 1 h of incubation were equivalent to 0.2 and 0.1 M, respectively. However, for the same cysteine concentration in the presence of patulin the SH groups at 1 h of incubation were equivalent to 0.06 and 0.04 M, respectively. The basal concentration of free sulfhydryl groups remained constant throughout the incubation in patulin-alone treatments with a mean concentration of 37.8 ± 2.64 mM (± SD).

DISCUSSION

In our experiments the toxin and the reducing agents were added at the same time to the in vitro rumen fermentations, simulating a field situation in which animals would receive a detoxifying compound top-dressed to contaminated feed. Patulin has a particular affinity for cellular thiol groups of proteins and glutathione (*18*), and the resulting adducts being formed are less toxic (*6*, *19*). This chemical reaction, which has been exploited for the detoxification of this mycotoxin in laboratory conditions, was unequivocally observed under the ruminal conditions tested, for example, presence of microorganisms, temperature, pH, and anaerobiosis. In the presence of thiol-containing reducing agents patulin disappeared from the incubation media and the toxic effects were not observed. Ascorbic

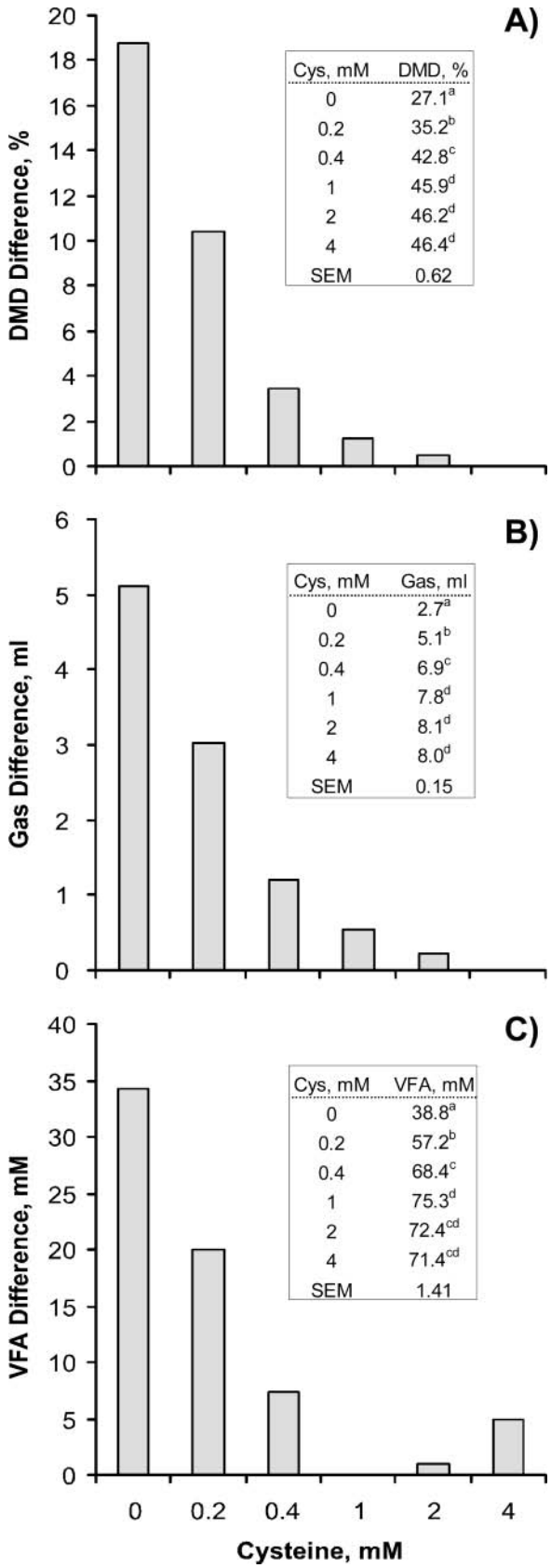

Figure 1. Prevention of patulin toxicity on in vitro rumen fermentation by cysteine. The negative effect of patulin on dry matter degradation (DMD) (**A**), gas (**B**), and volatile fatty acid (VFA) (**C**) was calculated as the difference between treatments with and without (50 μg/mL) patulin in the presence of increasing concentrations of cysteine. Insets show actual values for treatments containing patulin ($n = 6$). Means followed by different superscript letters differ ($P < 0.05$).

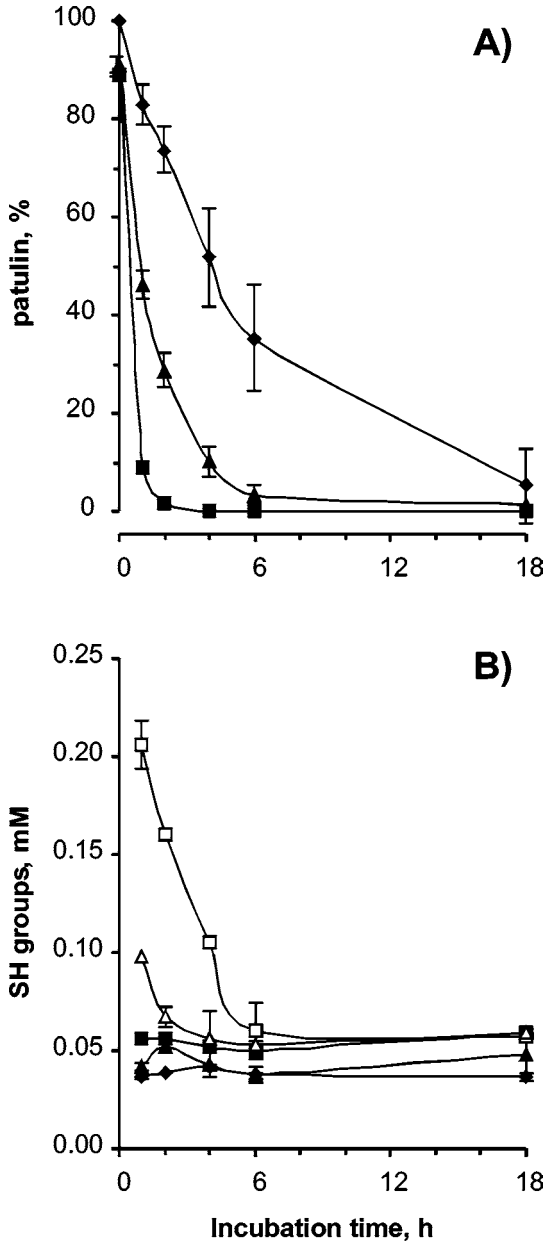

Figure 2. Changes in patulin (**A**) and SH groups (**B**) throughout 18 h of in vitro rumen fermentation in the presence of 0 (◆), 0.2 (▲), and 0.4 (■) mM cysteine. Open symbols (△, □) in panel **B** represent 0.2 and 0.4 mM cysteine in the absence of patulin (50 μg/mL). Results are expressed as means ± SD ($n = 3$).

acid also inactivates patulin in solutions, and its use has been explored for the decontamination of apple juice (*20*). However, in our system the addition of ascorbic acid was not effective in reducing the negative effects of the toxin on rumen fermentation. The degradation of patulin by ascorbic acid is slow (*8*, *9*), and it was certainly the reason for the lack of effect of this reducing agent.

The instability of patulin in the in vitro fermentations suggests that the rumen naturally acts as a protective barrier against toxicological damage to ruminant tissues. In monogastric animals, patulin produces ulcerative lesions and inflammation at the gastrointestinal tract level due to its toxicity on the epithelia (*21*−*24*). In ruminants, the rumen barrier will limit the injury on gastrointestinal epithelium and other organs. Mild intoxications will mainly affect the efficiency of fermentation in the rumen. In contrast to the toxin's lack of stability, patulin seemed to be more toxic to rumen microbes than previously reported. The toxin had a marked negative effect on rumen microbes at the minimal concentration of 25 μg/mL used in this study, which was similar to the concentration of 30 μg/mL reported to be toxic in continuous culture fermentors by Tapia et al. (*15*). However, in our experiments a reduced toxin concentration of ∼5 μg/mL detected at 1 h of incubation and not detectable at 2 h of incubation that was observed in treatments containing patulin and 0.4 M cysteine (**Figure 2A**) still affected negatively DMD, gas, and VFA production ($P <$ 0.05). Nevertheless, it has to be noted that the rumen inocula were not previously adapted to the toxin. Long-term exposure could increase the biological degradation of toxin or could induce resistance of rumen microbes to the antibiotic effects of patulin.

Patulin can be found occasionally at very high concentrations in corn silages. In a survey, ∼60% of samples were contaminated with this toxin at concentrations of up to 40 μg/kg (*1*). Patulin concentration in silage may decrease rapidly due to degradation by the producing organism (*25*), epiphytic yeasts (*26*), or chemical instability to high pH (*27*). This instability could be the reason for the scarcity of reports describing the presence of this toxin in field samples, although fungal strains capable of producing patulin are commonly isolated from silages (*13*, *27*). The fact that patulin is also not stable in the gastrointestinal tract of ruminants makes more difficult the diagnosis of natural intoxications. Due to the negative effect on rumen fermentation and the possible presence at high concentrations in silages patulin has the potential to affect production and health in cattle. Patulin's toxicity could be alleviated by the utilization of feed additives containing free SH groups such as cysteine.

SAFETY

Patulin has carcinogenic activity and was manipulated following appropriate safety precautions. Wastes were decontaminated following standard, published procedures (*8*, *28*).

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