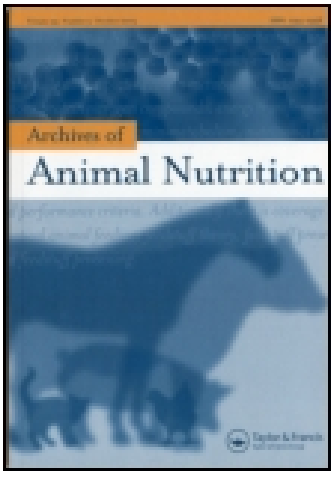

Archives of Animal Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gaan20

Effect of the combination of crude extracts of Penicillium griseofulvum and Fusarium graminearum containing patulin and zearalenone on rumen microbial fermentation and on their metabolism in continuous culture fermenters

María Belén Riccio[a], María Ofelia Tapia[a], Guadalupe Martínez[a], Sandra Mariela Aranguren[a], Susana Neyi Dieguez[a], Alejandro Luis Soraci[a] & Edgardo Rodríguez[a]

[a] Laboratorio de Toxicología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina

Published online: 30 Jun 2014.

To cite this article: María Belén Riccio, María Ofelia Tapia, Guadalupe Martínez, Sandra Mariela Aranguren, Susana Neyi Dieguez, Alejandro Luis Soraci & Edgardo Rodríguez (2014) Effect of the combination of crude extracts of Penicillium griseofulvum and Fusarium graminearum containing patulin and zearalenone on rumen microbial fermentation and on their metabolism in continuous culture fermenters, Archives of Animal Nutrition, 68:4, 309-319, DOI: 10.1080/1745039X.2014.927709

To link to this article: http://dx.doi.org/10.1080/1745039X.2014.927709

Archives of Animal Nutrition, 2014
Vol. 68, No. 4, 309–319, http://dx.doi.org/10.1080/1745039X.2014.927709

Taylor & Francis
Taylor & Francis Group

Effect of the combination of crude extracts of *Penicillium griseofulvum* and *Fusarium graminearum* containing patulin and zearalenone on rumen microbial fermentation and on their metabolism in continuous culture fermenters

María Belén Riccio, María Ofelia Tapia*, Guadalupe Martínez, Sandra Mariela Aranguren, Susana Neyi Dieguez, Alejandro Luis Soraci and Edgardo Rodríguez

Laboratorio de Toxicología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina

(*Received 1 July 2013; accepted 6 May 2014*)

Six single-flow continuous cultures were used to study the effects of the mycotoxins patulin (PAT) and zearalenone (ZEN) alone or in combination on rumen microbial fermentation. In each of the four 7-d periods, the fermenters were supplemented in a 2 × 3 factorial arrangement with two levels of PAT (0 and 20 mg/l) and three levels of ZEN (0, 5 and 10 mg/l). The treatments did not affect the apparent and true digestibility of organic matter. PAT alone decreased the digestibility of neutral detergent fibre (NDF) and acid detergent fibre (ADF) ($p < 0.01$), but in the presence of 5 or 10 mg/l of ZEN, there were no effects of PAT. In contrast, the digestibility of NDF and ADF was decreased at 10 mg/l of ZEN in the absence of PAT ($p < 0.05$). The pH of the fermenters increased after 2 and 3 d of PAT treatment ($p < 0.01$). PAT decreased the concentration of total volatile acids (VFA), the molar proportion of acetate and the acetate:proportionate ratio ($p < 0.01$). The molar concentrations of other VFA were unchanged. Ammonia N (NH_3–N) flow increased ($p < 0.05$) and there was a tendency to a higher NH_3–N concentration ($p < 0.1$) in fermenters with PAT. Total N, non-ammonia N and bacterial N as well as efficiency of microbial protein synthesis and efficiency of N utilisation were not affected by treatments. PAT was nearly completely degraded during incubation. The mean recovery of ZEN, α-zearalenol and β-zearalenol expressed as a proportion of administered ZEN was less than 50% in effluents from fermenters receiving only ZEN and ZEN plus PAT, respectively. With exception of fibre digestion, the co-administration of PAT and ZEN did not elicit interaction effects on most measured parameters of rumen metabolism.

Keywords: in vitro culture; mycotoxins; patulin; rumen metabolism; zearalenol; zearalenone

1. Introduction

Contamination of grain crops by fungi and their toxic metabolites, mycotoxins, is an important problem for livestock husbandry because of the adverse effects that it produces on animal health and productivity.

Experimental and field data on effects of mycotoxins on ruminants are highly variable and often contradictory. These discrepancies may be due to differences in the source of contamination (natural and purified) or experimental conditions. Observed effects of mycotoxins in ruminants have been reviewed several times (Seglar 1997; Fink-Gremmels 2008; Jouany et al. 2009). The co-occurrence of mycotoxins in animal feed

*Corresponding author. Email: tapia@vet.unicen.edu.ar

is also commonly reported (Streit et al. 2012). Thus, animals are generally not exposed to one but to several toxins at the same time. Grenier and Oswald (2011) reviewed more than one hundred publications on toxicological interactions of mycotoxins. They found that most of the studies reported synergistic or additive interactions regarding adverse effects on animal performance. This may be the reason why symptoms of mycotoxicosis are often seen in ruminants consuming feeds containing low concentrations of individual mycotoxins.

Since mycotoxins can have microbiostatic or microbicidal effects, it is possible that they interfere with rumen function to cause complications in ruminants which do not occur in other animals. For example, the microbial population could be shifted, ratios of end products of ruminal fermentation could be altered and digestion and intake by the ruminant could be impaired (Mertens 1978). However, toxicity of mycotoxins on rumen metabolism has been conclusively demonstrated for only a few mycotoxins. Acetic acid production was reduced in a batch culture exposed to 100 μg/ml of patulin (PAT; Escuola 1992), and *in vitro* dry matter (DM) and organic matter (OM) digestibilities were reduced in wheat straw to which 10 nmol of PAT had been added (Abdelhamid et al. 1992). Our group found that high doses of PAT adversely affected fermentation by ruminal microbes maintained in continuous culture fermenters (Tapia et al. 2002, 2005). In contrast, several studies suggested that ruminating animals are less susceptible to develop mycotoxicosis, as ruminal microorganisms may detoxify ingested mycotoxins. For instance, it has been postulated that ruminants are quite resistant to the estrogenic effects of zearalenone (ZEN) as the microbial rumen flora reduces its bioavailability (Kallela and Vasenius 1982). *In vitro* studies have demonstrated that ZEN is mainly degraded to α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) (Kiessling et al. 1984; Westlake et al. 1987; Macri et al. 2005).

Few published reports refer to the combined effects of Fusarium mycotoxins such as deoxynivalenol (DON) and ZEN on rumen metabolism (Dänicke et al. 2005; Seeling et al. 2006), and to our knowledge, combined effects of PAT and ZEN on rumen fermentation have not been studied. There is reason to speculate that the antimicrobial activity of PAT may result in an impairment of the rumen to metabolise ZEN. Therefore, the aim of this study was to investigate the interaction effects of crude extracts of *Penicillium griseofulvum* and *Fusarium graminearum*, containing mainly PAT and ZEN, respectively, on rumen microbial fermentation and on the outflow of PAT, ZEN and its metabolites α-ZEL and β-ZEL in continuous culture fermenters.

2. Materials and methods

2.1. Patulin source and analysis

Patulin was produced by growing *P. griseofulvum* NRRL 1953 in potato dextrose broth (Difco) at 28 ºC for 4 weeks. The mycelia and broth were then extracted twice with ethyl acetate. Extracts were pooled, evaporated to dryness in a rotary evaporator and reconstituted in chloroform. Extracts were kept at −18 ºC until use. Immediately prior to use, aliquots of the chloroform extract were evaporated to dryness under an N_2 stream, reconstituted in the mobile phase and then quantified using a Gilson HPLC system with a 151 UV/VIS detector. The column was a 150 mm × 4.60 mm reversed phase LUNA 5 μm C_8 (Phenomenex, Torrance, USA). The mobile phase was water: acetonitrile (95:5) at a flow rate of 1.5 ml/min. Patulin was detected at 276 nm.

Chloroform extracts were evaporated to dryness in a rotary evaporator. The dry residue was then reconstituted in distilled water immediately before addition to the continuous culture fermenters.

Patulin was extracted from effluents by a 2-step solvent extraction using ethyl acetate as organic solvent. Extracts were mixed and evaporated to dryness. Dried extract was reconstituted in MilliQ water and filtered through 0.22-µm nylon membranes before injection into HPLC.

2.2. Zearalenone source and analysis

Zearalenone was produced by growing *F. graminearum* NRRL 5884 on potato dextrose agar (Difco) at 25 ºC for 2 weeks and subsequently at 15 ºC for 3 weeks. Mycelia were extracted twice with alkaline water and chloroform. Extracts were pooled, evaporated to dryness in a rotary evaporator and then reconstituted in chloroform. Extracts were kept at −18 ºC until use. Immediately prior to use, aliquots of chloroform extract were evaporated to dryness under an N_2 stream, reconstituted in the mobile phase and quantified by HPLC with fluorescent detection. The column was a 250 mm × 4.60 mm reversed phase LUNA 5 µm C_{18} (Phenomenex, Torrance, USA). The mobile phase was water: acetonitrile (55:45) at a flow rate of 1.2 ml/min. Fluorescent detection (spectrofluorometric detector RF-10, Shimadzu, Kyoto, Japan) was at an excitation wavelength of 236 nm and an emission wavelength of 418 nm. Chloroform extracts were evaporated to dryness in a rotary evaporator. Dry residue was reconstituted in distilled water immediately before addition to the continuous culture fermenters.

Effluent samples were analysed for ZEN, α-ZEL and β-ZEL by HPLC with fluorescent detection after extraction with chloroform followed by cleaning of the extract with hexane. Chloroform extracts were evaporated to dryness, dissolved in mobile phase and quantified as described above. Retention times were 16, 6 and 10 min for ZEN, β-ZEL and α-ZEL, respectively.

2.3. Experimental design

Six 1000-ml single-flow continuous culture fermenters developed by Hannah et al. (1986) were used over four replicate periods of 7 d. Each experimental period consisted of 4 d for adaptation of the ruminal fluid to the continuous culture system with the last 3 d for mycotoxin addition and sample collection. Treatments were arranged in a 2 × 3 factorial design, the main factors being two levels of PAT (0 and 20 mg/l) and three levels of ZEN (0, 5 and 10 mg/l) in 1 ml of water every 8 h during the last 3 d.

Selection of the 20 mg/l dose of patulin was based on results from previous *in vitro* studies (Tapia et al. 2002, 2005) and levels of ZEN (5 and 10 mg/l) were in the range of doses reported to be estrogenic in dairy cattle (Mirocha et al. 1978; Weaver et al. 1986).

2.4. Continuous culture

On the first day of each period, fermenters were inoculated with fresh ruminal fluid from a cannulated cow fed a forage concentrate diet at 7:3 on DM basis. Diet was tested negative for aflatoxin B1 (limit of detection [LOD] 0.15 µg/kg), DON (LOD 20 µg/kg) and ZEN (LOD 4.5 µg/kg). Rumen fluid was collected before the morning feeding, strained through two layers of cheesecloth and kept in a thermos until inoculation into the fermenter flask. Fermenters were provided with 65.8 g DM of a ground diet, divided into three equal

portions at 08:00, 16:00 and 24:00 h. The diet contained per kg DM 380 g alfalfa hay, 280 g corn silage, 270 g cracked corn grain, 50 g soya bean meal and 6 g mineral mixture. The diet tested negative for patulin and ZEN.

The chemical composition of the diet was 918 g OM, 134 g crude protein (CP), 380 g neutral detergent fibre (NDF) and 230 g acid detergent fibre (ADF) on a DM basis. Flow rate of each fermenter was set at 0.06/h by regulating buffer input. Culture pH was recorded every 10 min by an electronic data acquisition system (Redimec, Tandil, Argentina). Anaerobic conditions in the fermenters were maintained by continuous infusion of N_2 at a rate 40 ml/min. The fermenter temperature was set at 38.5 ºC.

2.5. Sample collection

During sampling days, fermenter effluents were maintained in a 2 ºC water bath to retard microbial and enzymatic activities. On days 5, 6 and 7 of each period, fermenter effluents were homogenised for 5 min and three 500 ml samples were removed via aspiration. Upon completion of each period, effluents from the three sampling days were composited, mixed and homogenised for 5 min. Subsamples of effluents were collected for analysis of PAT, ZEN, α-ZEL and β-ZEL. Composite effluent samples were kept at −20 °C until analysis for volatile fatty acids (VFA), total N and ammonia N (NH_3–N). The remaining samples were lyophilised. Freeze dried composite samples were analysed for DM, OM, NDF, ADF and purines. At the end of each experimental period, the contents from fermenters flask were strained through two layers of cheesecloth, centrifuged at 1000 *g* for 10 min at 6–10 °C to remove feed particles and then the supernatant was centrifuged at 10, 000 *g* for 20 min at 6–10 °C to separate bacteria. Pellets were rinsed twice with saline solution and recentrifuged at 20,000 *g* for 20 min at 6–10 °C. The final pellets were resuspended in distilled water, frozen and lyophilised. Bacterial cells were analysed for DM, total N and purines. Purine contents of effluents and bacteria were used to partition effluent N flow into microbial and dietary N. Digestibility of DM, OM, fibre and CP and flows of total N, non-ammonia N (NAN), microbial N and dietary N were calculated as described by Stern and Hoover (1990).

2.6. Chemical analyses

The DM contents of effluents, diet and bacterial samples were determined by drying samples for 24 h in a 95 °C in a forced air oven and then were ashed overnight at 500 °C in a muffle furnace to determinate OM. Total N in the effluent, bacteria and diet was determined by a Kjeldahl method (976.05; AOAC 1990). For NH_3–N determination, orthophosphoric acid was added to the samples, centrifuged at 25,000 *g* for 20 min at 4 °C and then analysed by spectrophotometry (UV/VIS Spectrophotometer – Ultrospec III – Pharmacia LKB – Biochrom, England) (Chaney and Marbach 1962). Effluent samples for VFA were prepared as described by Jouany (1982) and analysis was by gas chromatography (Shimadzu, Model GC–17A, Kyoto, Japan) with a 19091N-133 Innowax 30M column (Agilent, Santa Clara, CA, USA). NDF and ADF expressed inclusive of residual ash in diet and effluent samples were analysed sequentially (Van Soest et al. 1991) using a thermo stable α-amylase and sodium sulphite with an ANKOM 200/220 fibre analyser (Van Soest et al. 1991).

Effluent and bacterial cells were analysed for purines by the method of Zinn and Owens (1986). Patulin, ZEN, α-ZEL and β-ZEL in effluents were analysed by the HPLC methods already described.

2.7. Statistical analysis

Data for rumen fermentation parameters were analysed as a completely randomised block design with a 2 × 3 factorial arrangement of treatments. Six treatment combinations were replicated four times. The total number of observation was 24. The analysis of variance used the GLM procedure of SAS. Treatment differences were declared significant when $p < 0.05$ and in those cases when significant interactions were observed, contrasts were used to compare the different levels of each treatment. Recovery of PAT in effluents was analysed as a completely randomised design. Recovery of α-ZEL and β-ZEL and ZEN in effluents from fermenters treated with 5 and 10 mg/l of ZEN was analysed as a completely randomised block design with a 2 × 2 factorial arrangement with two levels of PAT (0 and 20 mg/l).

3. Results

In fermenters supplemented with 20 mg/l of PAT, the pH increased during days 2 and 3 of mycotoxin addition ($p \leq 0.01$) (Table 1). But pH was not affected by addition of ZEN and no interaction between PAT and ZEN was observed.

Apparent and true digestibility of OM was not affected by PAT or ZEN treatments, but a mixture of PAT and ZEN showed an interaction ($p \leq 0.05$) on NDF and ADF digestibility (Table 1). In absence of ZEN, PAT decreased the digestibility of NDF and ADF ($p \leq 0.01$), but at 5 and 10 mg/l of ZEN, no effects of PAT were detected. On the other hand, a main effect of ZEN on was observed at 10 mg/l of ZEN in absence of PAT, where the digestibility of NDF and ADF was significantly decreased.

Patulin addition, alone or in combination with the highest addition of ZEN, induced a decrease in total VFA concentration ($p < 0.01$) (Table 2). Furthermore, the PAT addition decreased always the molar proportion of acetate ($p < 0.01$) and the acetate:propionate ratio ($p < 0.01$). This effect was independent of the level of ZEN. There were no interactions between PAT and ZEN on VFA production observed. The molar proportions of propionate, butyrate, valerate and branched-chain VFA were also not affected by treatments.

Table 1. Effects of patulin (PAT), zearalenone (ZEN) and their combination on pH and digestibility in continuous culture fermenters ($n = 4$ per group).

	ZEN [mg/l]									
	0		5		10					
	PAT [mg/l]							*p*-Value		
	0	20	0	20	0	20	SEM*	PAT	ZEN	PAT × ZEN
pH after mycotoxin addition										
Day 2	5.68	5.65	5.63	5.76	5.65	5.89	0.115	0.008	0.561	0.591
Day 3	5.61	5.70	5.63	5.80	5.61	5.90	0.101	<0.001	0.863	0.254
Digestibility [%]										
OM[◊] (apparent)	36.3	26.9	37.4	34.0	27.3	30.2	8.37	0.276	0.237	0.499
OM (true)[†]	43.0	33.2	43.7	40.1	34.8	38.3	7.98	0.242	0.327	0.399
NDF[‡]	28.9	8.0	19.8	18.3	10.9	17.8	6.79	0.408	0.145	0.011
ADF[♦]	32.8	9.0	22.2	17.8	12.8	20.4	7.64	0.845	0.286	0.027

Note: *SEM, Standard error of the mean; [†]Corrected for contribution of bacterial organic matter in the effluent; [◊]OM, Organic matter; [‡]NDF, Neutral detergent fibre; [♦]ADF, Acid detergent fibre.

Table 2. Effects of patulin (PAT), zearalenone (ZEN) and their combination on the concentration of volatile fatty acids (VFA) in continuous culture fermenters (n = 4 per group).

	ZEN [mg/l]									
	0		5		10					
	PAT [mg/l]							p-Value		
	0	20	0	20	0	20	SEM*	PAT	ZEN	PAT × ZEN
Total VFA [mmol/l]	127.2	116.0	120.5	122.9	147.6	119.5	11.30	0.006	0.517	0.442
Individual VFA [mmol/mol of total VFA]*										
Acetate	416.0	328.8	378.4	315.0	374.1	327	40.39	0.005	0.960	0.957
Propionate	247.5	316.0	276.4	286.2	278.1	289.3	30.47	0.121	0.407	0.154
Butyrate	279.2	294.4	291.0	349.3	281.5	327.4	41.87	0.613	0.238	0.429
Branch chain VFA	13.7	9.6	13.3	14.7	17.0	13.3	5.88	0.325	0.310	0.359
Valerate	28.0	34.9	25.9	24.9	34.1	29.6	4.69	0.241	0.397	0.311
Acetate: propionate	1.62	1.00	1.39	1.10	1.30	1.12	0.306	0.006	0.743	0.839

Note: *SEM, Standard error of the mean.

Total N, bacterial N and NAN flows and CP digestibility were not affected by either PAT alone or ZEN alone or in their combination (Table 3). In fermenters treated with PAT, NH_3–N flow increased ($p < 0.05$) and there was a tendency for a higher NH_3–N concentration ($p < 0.1$). The efficiency of bacterial synthesis (EMPS) expressed as grammes of bacterial N per kg of truly digested and the efficiency in N utilisation (ENU) were not affected by mycotoxin treatments (Table 3).

The total content of PAT in effluents as a proportion of PAT added to fermenters was 39.3, 35.7 and 34.5 g/kg in fermenters treated with 20 mg/l PAT alone or 20 mg/l PAT plus 5 or 10 mg/l of ZEN, respectively. The ZEN metabolites α-ZEL and β-ZEL were recovered at the effluents besides the parent toxin ZEN. The recovery of α-ZEL and β-ZEL and ZEN, expressed as percent of administrated ZEN (5 and 10 mg/l) is shown in Table 4.

4. Discussion

Mycotoxins interactions on different biological systems are reported in literature (Grenier and Oswald 2011). Nevertheless, combined effects of mycotoxins on rumen physiology have not received much attention and only few studies are reported.

Negative effects of PAT on *in vitro* rumen microbial fermentation are well documented (Tapia et al. 2002, 2005; Morgavi et al. 2003). On the other hand, it is postulated that ruminants are less sensitive to ZEN exposure as rumen microbes seem to degrade and hence to reduce the bioavailability of ZEN and its estrogenic potential (Kiessling et al. 1984; Westlake et al. 1987; Dänicke et al. 2005; Macri et al. 2005; Seeling et al. 2006). So, there was a reason to speculate that adverse effects of PAT on rumen microflora could modify rumen degradation of ZEN. In the present work the combined *in vitro* effects of PAT and ZEN on rumen microbial fermentation and on the flow of PAT, ZEN and ZEN were studied.

Table 3. Effects of patulin (PAT), zearalenone (ZEN) and their combination on ruminal nitrogen metabolism in continuous culture fermenters (n = 4 per group).

	ZEN [mg/l]									
	0		5		10					
	PAT [mg/l]							p-Value		
	0	20	0	20	0	20	SEM*	PAT	ZEN	PAT × ZEN
N intake [g/d]	1.69	1.71	1.72	1.70	1.70	1.71	0.012	0.140	0.394	0.065
Total N flow [g/d]	1.65	1.72	1.75	1.65	1.78	1.72	0.086	0.631	0.301	0.236
NH_3–N flow [g/d]	0.11	0.15	0.13	0.14	0.12	0.19	0.034	0.024	0.160	0.300
NAN[#] flow [g/d]	1.50	1.53	1.62	1.50	1.65	1.56	0.086	0.188	0.258	0.212
Dietary N flow [g/d]	1.06	1.18	1.25	1.11	1.18	1.10	0.100	0.311	0.453	0.050
NH_3–N concentration [mg/l]	69.0	84.6	74.6	86.5	75.2	107.5	20.07	0.060	0.267	0.511
Bacterial N [g/kg DM]	89.3	88.7	84.6	90.6	88.1	83.3	5.31	0.541	0.559	0.134
CP[◊] digestibility [%]	37	26	30	30	34	40	5.4	0.188	0.390	0.033
EMPS[†]	18.4	20.6	14.7	16.4	22.7	21.2	0.05	0.560	0.068	0.861
ENU[‡]	0.71	0.77	0.98	0.65	0.91	0.71	0.182	0.919	0.682	0.179

Notes: *SEM, Standard error of the mean; [#]NAN, Non-ammonia N; [◊]CP, Crude protein; [†]EMPS, Efficiency of microbial protein synthesis [g of bacterial N/kg of OM truly fermented in continuous culture]; [‡]ENU, Efficiency of N utilisation by ruminal bacteria, ENU = (microbial N [g]/ruminal available N [g]) · 100.

Table 4. Recovery of zearalenone (ZEN), α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) as proportion of administered ZEN.

	ZEN [mg/l]						
	5		10				
	PAT [mg/l]					*p*-Value	
	0	20	0	20	SEM	ZEN	PAT·ZEA
ZEN [%]	20.8	11.4	1.39	2.67	9.22	0.069	0.258
α-ZEL [%]	4.08	7.78	3.54	2.71	2.29	0.052	0.172
β-ZEL [%]	41.6	16.9	22.3	43.1	25.9	0.739	0.308

During the adaptation period before treatment, the pH in the fermenter varied from 5.65 to 5.88, but was significantly increased on days 2 and 3 of dosing 20 mg/l of PAT. Similar results have been reported by others (Morgavi et al. 2003; Tapia et al. 2005). It is postulated that the increase in pH was due to the decrease in VFA concentration in fermenters supplemented with PAT.

The apparent and true digestibility of OM was not affected by the PAT or ZEN treatments. These results disagree with previous studies reporting that 20 mg/l of PAT reduced the *in vitro* digestibility of OM (Tapia et al. 2002, 2005) and that 0.0015 mg/l of PAT reduced the digestibility of DM and OM of wheat straw *in vitro* (Abdelhamid et al. 1992). The low degradation of NDF and ADF observed in control fermenters observed in this study could be attributed to suboptimal pH (lower than 5.7) (Calsamiglia et al. 2002). Nevertheless, as in earlier reports (Tapia et al. 2002, 2005), a significant decrease in digestion of NDF and ADF in fermenters treated with PAT was found. A main effect on fibre digestibility was also observed with the highest dose of ZEN. The decrease in fibre digestibility could be attributed to a negative effect of both mycotoxins on cellulolytic bacteria activity. Antibacterial activity of PAT (Singh 1967; Escuola 1992) and ZEN (Boutibonnes 1979) has been demonstrated for Gram negative and Gram positive bacteria. Interestingly, the PAT and ZEN mixture did not affect fibre digestion. Further studies are necessary in order to give an insight into a possible interactive effect of these mycotoxins on fibre degradation and its biological meaning for the rumen metabolism.

The total VFA production and the acetate molar proportions in fermenters spiked with PAT alone or in combination with ZEN agree with previous reports (Morgavi et al. 2003; Tapia et al. 2005). As reported by Dänicke et al. (2005), VFA production and profile were unaltered by ZEN. The decrease in the acetate molar proportion may be attributed to a modification in the cellulolytic bacterial population, consistent with low digestibility of NDF and ADF.

As already reported by Tapia et al. (2005), in the present experiment there was an increase in NH_3–N flow and a tendency for a higher NH_3–N concentration in fermenters treated with PAT. Similarly, other *in vitro* fermentation studies using long-term simulation (Rusitec) demonstrated a 68% and 35% increase in NH_3–N concentrations for mouldy corn silage and mouldy grass, respectively (Maiworm et al. 1995; Holtershinken et al. 1997). Ammonia in fermenters is derived from degradation of dietary protein and dietary non-protein N. On the other hand, the main cellulolytic bacterial species use NH_3 as their main source of N for microbial protein synthesis; thus, 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 study, the increase in NH_3–N flow and NH_3–N concentration, together with no changes in the total production of bacterial N suggest a reduction in the activity and in the growth of cellulolytic bacteria with a shift in the bacterial community population in fermenters treated with PAT.

The metabolism of ingested material by the ruminal microbes may be considered as a first line of defence against harmful substances present in the diet. In the present study, the flow of PAT at the effluents ranged from 3.45% to 3.93% of PAT addition. These findings suggest an intensive ruminal metabolism of PAT, which resulted in a substantial disappearance of this mycotoxin. These results are in agreement with observation of Morgavi et al. (2003), who reported that PAT was almost not detected after a 18-h incubation period. However, PAT might not only undergo a metabolism in the rumen, but could exhibit antimicrobial activity as this mycotoxin affects the fermentative capacity of the rumen such as digestion of OM and fibre and production of bacterial end products (Tapia et al. 2002, 2005; Morgavi et al. 2003). Degradation of PAT was not modified by ZEN.

No significant differences were observed in ZEN disappearance in fermenters treated with two levels of ZEN alone or in combination with PAT. After 3 days of treatment, ZEN recovery from effluents ranged from 1.39% to 20.8% of the ZEN intake. In previous *in vitro* experiments with rumen fluid, a minor degradation of ZEN had been reported by other authors. Kallela and Vasenius (1982) showed that after 48 h of incubation with bovine rumen fluid the ZEN content decreased by on average 37.5%. In another study, Macri et al. (2005) reported that ZEN was degraded to 63% and 49% of the initial concentration within 24 h when incubated with solid and fluid rumen digesta, respectively.

The mean recovery of ZEN, α-zearalenol and β-zearalenol expressed as a proportion of administered ZEN was less than 50% in effluents from fermenters receiving only ZEN and ZEN plus PAT, respectively. Similarly, one *in vitro* fermentation study (Rusitec) by Seeling et al. (2006) showed that the mean recovery of ZEN and α-ZEL expressed as percentage of administered ZEN in effluent amounted to 55%. However, in contrast to this and to a previous experiment (Kiessling et al. 1984), where α-ZEL was the main metabolite, whereas β-ZEL was produced in small proportion or below the detection limit, in our study ZEN was mainly reduced to β-ZEL and to a lesser degree to α-ZEL. As expressed by Seeling et al. (2006), it is possible that differences in experimental conditions could be related to differences in the extent of ZEN metabolism to α-ZEL and β-ZEL. This view is supported by unpublished *in vitro* studies carried out in our laboratory in which batch cultures instead of the continuous culture system were used to describe ruminal metabolism of ZEN. In this study, ZEN concentration in rumen fluid decreased linearly throughout the 48-h incubation period, while there was a linear increase of α-ZEL concentration and β-ZEL was below the detection limit of the analytical method.

Whether the ruminal metabolism modifies the estrogenic potential of ZEN remains unclear and deserves further investigation. Besides, it is important to consider the influence of ZEN and metabolites with regard to possible antimicrobial activity on rumen microorganisms and thereby on rumen metabolism.

5. Conclusions

This study corroborated earlier findings indicating that PAT affects certain aspects of rumen fermentation such as fibre digestibility and VFA production. In addition, some effect of ZEN on fibre digestibility was observed. Moreover, the low recovery of PAT at

the effluent indicated a nearly complete degradation of the molecule in fermenters, which was not modified by ZEN. On the other hand, the adverse effects of PAT on rumen fermentation did not affect rumen metabolism of ZEN.

References

Abdelhamid AM, El-Ayouty SA, El-Saadany HH. 1992. The influence of contamination with separate mycotoxins (aflatoxins, ochratoxin A, citirnin, patulin, penicillic acid or sterigmatocystin) on the in vitro dry matter and organic matter digestibilities of some roughages (berseem hay and wheat straw). Arch Anim Nutr. 42:179–185.

AOAC. 1990. Official methods of analysis. 15th ed. Arlington (VA): Association of Official Analytical Chemists.

Boutibonnes P. 1979. Effets de la zearalenona sur la cellule bacterienne. Mycopatholia. 69:117–120.

Calsamiglia S, Ferret A, Devant M. 2002. Effects of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. J Dairy Sci. 85:574–579.

Chaney AL, Marbach EP. 1962. Modified reagents for determination of urea and ammonia. Clinical Chem. 8:130–132.

Dänicke S, Matthäus K, Lebzien P, Valenta H, Stemme K, Ueberschär KH, Razzazi-Fazeli E, Böhm J, Flachowsky G. 2005. Effects of *fusarium* toxin-contaminated wheat grain on nutrient turnover, microbial protein synthesis and metabolism of deoxynivalenol and zearalenone in the rumen of dairy cows. J Anim Physiol Anim Nutr. 89:303–315.

Escuola L. 1992. Patulin production by *penicillium granulatum* and inhibition of ruminal flora. J Environ Pathol Toxicol Oncol. 11:109–112.

Fink-Gremmels J. 2008. The role of mycotoxins in the health and performance of dairy cows. Vet J. 176:84–92.

Grenier B, Oswald IP. 2011. Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. World Mycotoxin J. 4:285–313.

Hannah SM, Stern M, Ehle FR. 1986. Evaluation of a dual flow continuous culture system for estimating bacterial fermentation in vivo of mixed diets containing various soya bean products. Anim Feed Sci Technol. 16:51–62.

Holtershinken M, Plitt U, Tammen FC, Hoffmann P, Scholz H. 1997. Influence of moldy grass on fermentation and thiamine metabolism in bovine rumen fluid (in-vitro). Dtsch Tierärztl Wschr. 104:17–22.

Jouany JP. 1982. Volatile fatty acids and alcohol determination in digestive contents, silage juice, bacterial cultures and anaerobic fermentor contents. Sci Alim. 2:131–144.

Jouany JP, Yiannikouris A, Bertin G. 2009. Risk assessment of mycotoxins in ruminants and ruminant products. Options Mediterranéennes, A. 85:205–224.

Kallela K, Vasenius L. 1982. The effects of rumen fluid on the content of zearalenone in animal fodder. Nord Vet Med. 34:336–339.

Kiessling K, Pettersson H, Sandholm K, Olsen M. 1984. Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichotecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. Appl Environ Microbiol. 45:1070–1073.

Macri A, Schollenberger M, Drochner W, Tafaj M, Morar V. 2005. Investigation on the "*in vitro*" degradation of zearalenone in rumen fluid. Mycotoxin Res. 21:65–67.

Maiworm VK, Holtershinken M, Scholz H. 1995. The effects of mouldy and decomposed silage on rumen. Tierärztl Umschau. 50:283–290.

Mertens DR. 1978. Biological effects of mycotoxins upon rumen function and lactating dairy cows. In: Interaction of mycotoxins in animal production. Proceedings of a symposium; Jul 13. Washington (DC): National Academy of Sciences, Michigan State University; p. 118–136.

Mirocha CJ, Weaver G, Gustafsson B, Chi M, Pathre SV, Robinson TS, Bates F. 1978. Pharmacological and toxicological studies on zearalenone in food producing animals. Quarterly Report 11. Washington, DC, USA: FDA, 125 p.

Morgavi DP, Boudra H, Jouany JP, Graviou D. 2003. Prevention of patulin toxicity on rumen microbial fermentation by sh-containing reducing agents. J Agric Food Chem. 51:6906–6910.

Seeling K, Boguhn J, Strobel E, Dänicke S, Valenta H, Ueberschär KH, Rodehutscord M. 2006. On the effects of fusarium toxin contaminated wheat and wheat chaff on nutrient utilisation and turnover of deoxynivalenol and zearalenone in vitro (Rusitec). Toxicol in Vitro. 20:703–711.

Seglar B. 1997. Case studies that implicate silage mycotoxins. In: Silage: Field to feedbunk. Hershey (PA): Northeast Regional Agricultural Engineering Service; p. 242–254.

Singh J. 1967. Patulin. In: Gotlieb D, Shaw PD, editors. Antibiotics. Mechanisms of action. New York: Springer-Verlag; p. 621–630.

Stern MD, Hoover WH. 1990. The dual flow continuous culture system. In: Miner WH, editor. Continuous Culture Fermenters: Frustration or Fermentation? Workshop Handbook for NE ADSA ASAS Regional Meeting; Jul 8; Chazy, NY, p. 17–32.

Streit E, Schatzmayr G, Tassis P, Tzika E, Marin D, Taranu I, Tabuc C, Nicolau A, Aprodu I, Puel O, Oswald IP. 2012. Current situation of mycotoxin contamination and co-occurrence in animal feed - focus on Europe. Toxins. 4:788–809.

Tapia MO, Stern MD, Koski RL, Bach A, Murphy MJ. 2002. Effects of patulin on rumen microbial fermentation in continuous culture fermenters. Anim Feed Sci Technol. 97:239–246.

Tapia MO, Stern MD, Soraci AL, Meronuck R, Olson W, Gold S, Koski-Hulbert R, Murphy MJ. 2005. Patulin-producing molds in corn silage and high moisture corn and effects of patulin on fermentation by ruminal microbes in continuous culture. Anim Feed Sci Technol. 119:247–258.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3583–3597.

Weaver GA, Kurtz HJ, Behrens JC, Robison TS, Seguin BE, Bates FY, Mirocha CJ. 1986. Effect of zearalenone on the fertility of virgin dairy heifers. Am J Vet Res. 47:1395–1397.

Westlake K, Mackie RI, Dutton MF. 1987. Effects of several mycotoxins on specific growth rate of *butyruvibrio fibrisolvens* and toxin degradation in vitro. Appl Environ Microbiol. 53:613–614.

Zinn RA, Owens FN. 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can J Anim Sci. 66:157–166.