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Residues of zearalenone (ZEN), deoxynivalenol (DON) and their metabolites in plasma of dairy cows fed *Fusarium* contaminated maize and their relationships to performance parameters

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ABSTRACT

A feeding trial with dairy cows fed graded proportions of a *Fusarium* toxin contaminated maize containing mainly deoxynivalenol (DON) was carried out to relate the plasma levels of DON, zearalenone (ZEN) and their metabolites to the performance. German Holstein cows (n = 30) were divided into three groups (n = 10 in each): CON (0.02 mg ZEN and 0.07 mg DON, per kg dry matter, DM), FUS-50 (0.33 mg ZEN and 2.62 mg DON, per kg DM), FUS-100 (0.66 mg ZEN and 5.24 mg DON, per kg DM).

The average performance level was characterised by daily DM intake, energy balance and milk yield which were not affected by the DON and ZEN levels in feed. DON, de-epoxy-DON (de-DON) and ZEN were detected simultaneously in all plasma samples. A linear relationship between toxin intake and plasma levels could be established. Moreover, a linear relationship between DON and de-DON concentration could be derived. It was concluded that DON and ZEN intake of 0.5 mg ZEN/kg and 5 mg DON/kg (current guidance values) had no considerable effects on the performance parameter of dairy cows. Furthermore, increased plasma concentrations of ZEN, DON and de-DON may hint on toxin exposure through the diets.

1. Introduction

Silage of whole plant maize represents a high-energy feed component of dairy cow rations. Under central European cultivation conditions maize is often infested by different moulds of the genus *Fusarium*, whereby *F. graminearum* and *F. verticillioides* are most important (Miedaner et al., 2010). Their toxin-forming ability is of particular importance depending on the genotype of maize and the phytopathogeny of various fungal isolates. The toxins zearalenone (ZEN) and deoxynivalenol (DON) are found in concentrations, which can be toxicologically relevant for farm animals as guidance values for critical feed concentrations might be exceeded (Döll and Dänicke, 2011).

The European Food Safety Authority (EFSA) requires for dose–response studies to improve the data base for ruminants (EFSA, 2004a, b) and to substantiate the established guidance values for animal feed (2006/576/EG, 2006). Currently, the guidance values are given with 0.5 mg ZEN/kg and 5 mg DON/kg relative to feed with 12% moisture content.

Only a few studies (Dänicke et al., 2002; Keese et al., 2008b; Seeling et al., 2005; Seeling et al., 2006a) examined the effects of ZEN and DON on health and performance and on toxin residues in blood as an indicator for the internal exposure at the same time.

Some negative effects were observed with regard to feed intake and milk composition (Charmley et al., 1993; Trenholm et al., 1985). In a field study cows received a spoiled acid-treated maize with 1.5 mg ZEN and 1 mg DON/kg resulting in poor feed consumption, depressed milk production and diarrhea (Coppock et al., 1990). Whitlow and Hagler (1999) suggested that DON could serve as a marker for feed which was exposed to *Fusarium* mould growth and mycotoxins formation. In consequence, the possible

Abbreviations: 15-ADON, 15-acetyl-DON; α-ZAL, α-zearalanol; β-ZAL, β-zearalanol; α-ZEL, α-zearalenol; β-ZEL, β-zearalenol; BCS, body condition score; BW, body weight; CON, control group; DAD, diode array detector; de-DON, de-epoxy-DON; DM, dry matter; DON, deoxynivalenol; DON-3G, DON-3-glucoside; EFSA, European Food Safety Authority; F., Fusarium; FCM, fat corrected milk; FUS-50, group which received a contaminated diet (0.25 mg ZEN/kg and 2.5 mg DON/kg DM); FUS-100, group which received a contaminated diet (0.5 mg ZEN/kg and 5 mg DON/kg DM); HPLC, high-performance liquid chromatography; IAC, immune-affinity column; LC, liquid chromatography; LOD, limit of detection; LS, least square; MS, mass spectrometry; NEL, net energy lactation; SE, standard error; TMR, total mixed ration; ZAN, zearalanone; ZEN, zearalenone.

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presence of other mycotoxins or toxic factors more toxic than DON itself should be deduced.

In other studies, both ZEN and DON were investigated individually (Charmley et al., 1993; Keese et al., 2008b; Seeling et al., 2005). However, as ZEN and DON often co-occur in feedstuffs a diagnostic opportunity to evaluate the internal exposure to both toxins at the same time would be helpful. Additionally, the analytical methods used until now can hardly detect DON and ZEN together with their metabolites in bovine serum due to the detection limits which were too high for an appropriate evaluation of exposure to the toxins. Therefore, a new LC–MS/MS method coupled with a solid phase extraction (SPE) was applied to reduce the detection limits and to increase the probability to detect ZEN, DON and their metabolites in different body fluids like plasma.

In order to generate blood samples with increasing concentrations of both toxins and their metabolites, a dose–response study was carried out with dairy cows. A control ration and two rations with increasing concentrations of ZEN and DON contaminated maize were tested. From a diagnostic perspective possible relationships between plasma toxin levels and toxin effects on performance were also of interest. The guidance value of 0.5 mg ZEN/kg diet and 5 mg DON/kg diet were targeted as a basis for the highest dietary concentration.

2. Materials and methods

2.1. Animals, diets and experimental procedure

The study was conducted at the experimental station of the Institute of Animal Nutrition in Braunschweig, Germany with a total of 30 lactating German Holstein cows which were divided into three groups ($n = 10$ in each). All three groups consisted of four heifers and six pluriparous cows. All rations were fed as a total mixed ration (TMR), which consisted of 50% grass silage and 50% concentrate on DM basis (Tables 1 and 2). Ten cows received a control diet (control group (CON), 50% grass silage and 50% control concentrate on dry matter (DM) basis) and twenty cows received a *Fusarium* toxin-contaminated diet. These animals were divided into FUS-50 group (50% grass silage, 25% CON concentrate and 25% FUS concentrate) and FUS-100 group (50% grass silage and 50% FUS concentrate) (Tables 1 and 2). The toxin contamination of the whole ration was achieved by the exchange of control maize with contaminated maize grains (3.2 mg ZEN/kg; 39 mg DON/kg) and contaminated maize cobs (48 mg ZEN/kg; 299 mg DON/kg). The contaminated maize plants were obtained by artificial cob inoculation with *F. graminearum* which was described in detail by Rempe et al. (2013). The intended DON and ZEN contents in the rations amounted to 0.25 mg ZEN/kg and 2.5 mg DON/kg DM for the FUS-50 group, and to 0.5 mg ZEN/kg and 5 mg DON/kg DM for the FUS-100 group.

Table 1
Components, energy content and composition of the concentrates ($n = 2$) and the grass silage.

	Concentrate		Grass silage
	CON	FUS	
Components (%)			
Dried beet pulp	30.7	30.7	
Barley	21.0	20.0	
Rapeseed extraction meal	20.0	20.0	
Maize	20.0		
Fusarium toxin contaminated maize		20.0	
Soybean extraction meal	6.5	6.5	
Fusarium toxin contaminated maize cobs		1.0	
Mineral feed[c]	1.8	1.8	
DM[a] (%)	88	88	42
NEL[b] (MJ/kg DM)	7.70	7.60	5.89
Nutrient composition (g/kg DM)			
Crude ash	61	61	92
Crude protein	184	186	133
Crude fat	34	29	35
Crude fibre	107	107	279
Acid detergent fibre	141	145	293
Neutral detergent fibre	282	286	496
Calculated mycotoxin composition (mg/kg DM)			
DON	0	9.8	
ZEN	0	1.0	
Mycotoxin composition (mg/kg DM)			
DON	0.13	10.47	0.00
ZEN	0.04	1.31	0.01

[a] DM, dry matter.
[b] NEL, net energy lactation.
[c] Per kg mineral feed: 170 g Ca; 120 g Na; 50 g P; 45 g Mg; 6 g Zn; 5 g Mn; 1.3 g Cu; 120 mg I; 40 mg Se; 35 mg Co; 800,000 IE vitamin A; 100,000 vitamin D_3; 4 g vitamin E.

Table 2
Energy content and composition of the TMR.

Group	CON	FUS-50	FUS-100
DM (%)	48	53	50
NEL (MJ/kg DM)	6.80	6.77	6.75
Nutrient composition (g/kg DM)			
Crude ash	86	81	81
Crude protein	163	159	160
Crude fat	35	28	28
Crude fibre	201	194	196
Acid detergent fibre	230	217	226
Neutral detergent fibre	420	409	420
Calculated mycotoxin composition (mg/kg DM)			
DON	0	2.45	4.90
ZEN	0	0.25	0.51
Mycotoxin composition (mg/kg DM)			
DON	0.19	1.78	3.97
ZEN	0.03	0.28	0.63

The feeding experiment covered a period of 13 weeks, starting at 7th day *postpartum*. During the study cows were housed in group pens according to their feeding group. The pens were equipped with slatted floors and cubicles equipped with rubber mattresses and wood litter.

Samples of concentrates and TMR were collected weekly and pooled over four weeks. For further analysis the samples were dried at 60 °C and ground to pass a 1 mm sieve. TMR and water were offered *ad libitum* from self-feeding stations (Type RIC, Insentec, B.V., Marknesse, the Netherlands). The daily individual feed and water intake was recorded with ear transponders.

The cows were milked twice a day and milk yield was determined each time. Additionally, the body weights (BW) were measured automatically after leaving the milking parlour. Moreover, milk samples for the analysis of milk composition were taken twice a week in the morning and in the afternoon of the same day. After conserving with bronopol, the samples were stored at 8 °C until analysis. Furthermore, the body condition score (BCS) of each cow was determined according to Edmonson et al. (1989).

Blood samples were collected by puncture of one *Vena jugularis externa* one day before the feeding trial started, after one week, at week nine and at the end of the feeding trial from each cow. Plasma was separated by centrifugation at 3000 *g* for 15 min at 15 °C and kept at −20 °C immediately until mycotoxin residue analysis.

Experiment and procedures were conducted according to Animal Welfare Act with the approval of the Lower Saxony State Office for Consumer Protection and Food Safety of Oldenburg, Germany.

2.2. Analysis

The nutrient composition of both concentrates and TMR was analysed according to the methods of the Association of German Agricultural Analytic and Research Institutes (VDLUFA) (Bassler, 1976).

ZEN in feed was analysed by HPLC with fluorescence detection after clean-up with IAC (ZearalaTest WB, Vicam, Milford, MA, USA) according to a slightly modified method of VDLUFA, 2006. DON in feed was determined by high-performance liquid chromatography (HPLC) with diode array detector (DAD) after clean-up with immuno-affinity columns (IAC, DONprep, R-Biopharm AG, Darmstadt, Germany) according to Oldenburg et al. (2007).

For the toxin analysis, 500 µL plasma was spiked with internal standard (250 ng/mL for $^{13}C_{18}$-ZEN, α-ZEL-d_4, β-ZEL-d_4, α,β-ZAL-d_4 and 500 ng/mL for $^{13}C_{15}$-DON) and 1 mL sodium acetate buffer (pH 5.5) and 20 µL β-glucuronidase (Type H-2 from *Helix pomatia*; Sigma–Aldrich; Steinheim, Germany) were added. Before clean-up by solid phase extraction (Oasis HLB, Waters, USA) the sample was incubated over night at 37 °C. The sample preparation was performed according to a modified method of Brezina et al. (2013). Afterwards, the analytes ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), DON and de-epoxy-DON (de-DON) were determined by LC–MS/MS which consist of an Agilent 1200 series (Agilent Technologies, Böblingen,