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Research Article

No carry over of unmetabolised deoxynivalenol in milk of dairy cows fed high concentrate proportions

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To examine the carry over of deoxynivalenol (DON) and its metabolite de-epoxy DON (DOM-1) in milk, lactating German Holstein cows (n = 13) were fed an isoenergetic total mixed ration in Period 1 with 50% concentrates and 5.3 mg DON/kg dry matter (DM) over 11 wk and were compared with control cows (n = 14). In Period 2 (18 wk), an elevated concentrate proportion was compared to a low concentrate ration by dividing the cows into four Groups (n = 8): Control-30 (30% concentrates), Myco-30 (30% concentrates, 4.4 mg DON/kg DM), Control-60 (60% concentrates) and Myco-60 (60% concentrates, 4.6 mg DON/kg DM). Taken both periods together, no unmetabolised DON was detected in milk samples using the HPLC-UV method. DOM-1 concentrations ranged between below the LOD and 3.2 μg/kg milk in mycotoxin fed cows, while control cows did not excrete any measurable amounts of DOM-1. Regarding the concentrate effects, the carry over of DON as DOM-1 in milk was negligible (between 0.0001 and 0.0011) but significantly higher in Group Myco-30 than in Group Myco-60. This effect may result from an altered bioavailability of DON from maize silage which made up a higher proportion of the daily ration.

Keywords: Carry over / Dairy cow / Deoxynivalenol / *Fusarium* mycotoxin / Milk

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1 Introduction

In Europe, deoxynivalenol (DON), a secondary metabolite (mycotoxin) of moulds of various *Fusarium* species, is frequently found in toxicologically relevant concentrations for farm animals [1]. Ruminants are regarded as relatively resistant to toxic effects of DON due to the potential of its ruminal microbes to degrade DON to the less toxic metabolite de-epoxy DON (DOM-1), and the carry over from DON in milk is generally regarded as negligible [2 – 4]. In previous studies, DOM-1 was detected in blood, duodenal chyme, urine, faeces and milk mostly in higher concentrations than the parent toxin [2, 5 – 8].

The intact ruminal epithelium was shown to be an effective barrier for DON and zearalenone (ZON) [9]. However, Sabater Vilar [10] found very high concentrations of unmetabolised DON (up to 58 μg/L) in the serum of dairy cows showing an increased mastitis incidence at the same time. DON in milk was not determined. In serum, DON was detected by an ELISA method, but no applicability of the applied method was provided by the authors. The estimated daily DON intake was, with 4.5 mg, rather low, but the authors concluded that the rumen was not able to metabolise this amount of DON. Unpublished reports about high concentrations of unmetabolised DON in the serum of clinically affected dairy cows raise the question what factors influence the degradation of DON and if disease-related alterations in the ruminal environment and/or ruminal mucosa may lead to an elevated absorption of the parent toxin in the blood circulation and therewith connected an elevated carry over rate in milk.

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Abbreviations: BW, body weight; **DM,** dry matter; **DMI,** dry matter intake; **DOM-1,** de-epoxy DON; **DON,** deoxynivalenol; **IAC,** immunoaffinity column; **LSmeans,** least square means; **OA,** ochratoxin A; **SARA,** subacute ruminal acidosis; **TMR,** total mixed ration; **ZON,** zearalenone

Table 1. Composition of the concentrates ($n = 1$) and silages ($n = 2$, means) used in Period 1

	Control concentrate	Myco concentrate	Maize silage	Grass silage
Components (%)				
Triticale	50	21		
Fusarium-contaminated triticale	0	29		
Soyabean meal	26.7	26.7		
Maize	20.7	20.7		
Mineral feed[a)]	1.4	1.4		
Calcium carbonate	1.2	1.2		
DM (g/kg)	866	867	354	335
Nutrients (g/kg DM)				
Crude ash	57	57	37	93
Crude protein	195	201	87	123
Crude fat	26	28	26	24
Crude fibre	34	35	188	316
Acid detergent fibre	47	47	205	328
Neutral detergent fibre	131	135	396	561
Mycotoxins				
Deoxynivalenol (mg/kg DM)	0.2	9.6	1.8	<0.03
ZON (μg/kg DM)	9.8	129.0	191.2	1.7

Period 1, week 1 – 11; Control, cows fed the control diet with 50% concentrate (based on DM); Myco, cows fed the *Fusarium* toxin-contaminated diet with 50% concentrate (based on DM).

a) *Per* kg mineral feed: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu; 100 mg I; 40 mg Se; 25 mg Co; 1 000 000 IU vitamin A; 100 000 IU vitamin D_3; 1500 mg vitamin E.

It was shown for ochratoxin A (OA) that the concentrate proportion significantly influences the degradation of OA to the less toxic metabolites ochratoxin α (Oα) and phenylalanine [11 – 13]. Sheep that consumed 14 μg OA/kg body weight (BW) from a ration containing 70% concentrate showed a decreased ruminal pH postfeeding, a delayed OA disappearance from the rumen and an increased OA concentration in blood compared to sheep fed 30% concentrates [13]. Accordingly, He *et al*. [14] reported about a pH-dependent biotransformation of DON in the large intestines of chicken, which was completely inhibited by pH values below 5.2.

The possible influence of the composition of the diet on the metabolism of DON in ruminants has not been examined sufficiently. In rations for high yielding dairy cows, high concentrate proportions of 60% are used which can lead to a condition described as subacute ruminal acidosis (SARA) with decreased ruminal pH values down to 5.5 – 5.6 [15 – 17], adaptive changes up to degenerative processes in the ruminal mucosa and changed ruminal conditions [18 – 20]. In this situation, the absorption of bacteria causing necrosis and pus, primarily in the liver, were facilitated through the impaired ruminal mucosa [21].

Therefore, the aim of the present study was to investigate whether unmetabolised DON can pass the ruminal epithelium; whether it appears in various biological substrates including milk in cows fed a diet with a high concentrate proportion, and whether the metabolite profile of DON is altered compared to cows fed a low concentrate diet.

This paper is a part of a comprehensive study in which *Fusarium* toxin-contaminated triticale was fed to lactating dairy cows in rations with 30, 50 and 60% concentrate. In this paper, the focus is on the carry over of DON and DOM-1 in milk and the DON residues in serum and bile in *Fusarium* toxin-exposed lactating cows, while effects on performance parameters, ruminal fermentation and parameters of the acid – base metabolism are discussed at Keese *et al*. [22, 23].

2 Materials and methods

2.1 Treatments, experimental design and animals

The experimental design is described in more detail by Keese *et al*. [22]. In brief, in Period 1, 27 lactating German Holstein cows (mean BW = 522 ± 56 kg, mean days in milk (DIM) = 31 at the beginning of the trial) were divided into two groups for a duration of 11 wk. A total of 14 animals (Control Group) received the Control diet (50% concentrate, 25% maize silage and 25% grass silage on dry matter (DM) basis) and 13 animals (Myco Group) received the *Fusarium* toxin-contaminated diet (mean DON concentration of 5.3 mg/kg DM; 50% concentrate, 25% maize silage and 25% grass silage) (Tables 1 and 2).

Directly after finishing Period 1, the same 27 cows plus 5 additional cows were divided into 4 feeding groups. Cows fed the control diet in Period 1 also received the control diets in Period 2. Animals that were fed the *Fusarium* toxin-

Table 2. Composition of the TMR used in Period 1 ($n = 1$)

Group	Control	Myco
DM (g/kg)	452	465
Nutrients (g/kg DM)		
Crude ash	61	61
Crude protein	150	153
Crude fat	25	26
Crude fibre	143	143
Acid detergent fibre	157	157
Neutral detergent fibre	305	307
Energy[a] (MJ/kg DM)		
ME	11.6	11.6
NEL	7.1	7.1
Mycotoxins[b]		
Deoxynivalenol (mg/kg DM)	0.6	5.3
ZON (μg/kg DM)	53.1	112.7

Abbreviations: Period 1, week 1 – 11; Control, cows fed the control diet with 50% concentrate (based on DM); Myco, cows fed the *Fusarium* toxin-contaminated diet with 50% concentrate (based on DM); ME, metabolisable energy; NEL, net energy lactation.

a) Calculation based on formulas of the GfE [47].

b) Calculation based on analysed concentrations in concentrates and silages.

contaminated diet in Period 1 were fed the *Fusarium* toxin-contaminated diets in Period 2 as well. Because of a later calving date, the five additional cows were not available for Period 1. Two of them were used as control animals and had received the same diet as the Control Group for 8 wk before Period 2 started; the other three animals were fed the same diet as the Myco Group for 8 wk as well.

In Period 2, the concentrate proportion in two groups was elevated to 60% in the presence and absence of *Fusarium* toxin-contaminated triticale (Group Control-60 and Group Myco-60), and the effects were compared to two low level concentrate groups (30% concentrates on DM basis; Group Control-30 and Group Myco-30) (compare Tables 3 and 4). The DON concentration was, with 4.8 mg/kg DM (on a DM basis of 88%), calculated to be similar in Group Myco-30 and Group Myco-60 and similar to the DON concentration used in Period 1, so that the effects of different concentrate levels could be examined.

Directly after finishing the last week of feeding the *Fusarium* toxin-contaminated diets all cows received the control ration with 30% concentrates (Control-30) for another 3 wk (depletion period).

During both periods all groups were housed in group pens equipped with slatted floors and cubicles equipped with rubber mattresses and wood litter. All rations were fed as a total mixed ration (TMR) for *ad libitum* intake from self-feeding stations (Type RIC, Insentec, B.V., Marknesse, The Netherlands). The daily individual feed intake was recorded by using an ear transponder for each cow. For technical reasons only the total feed intake of both groups was documented daily in Period 1. Freshly prepared feed was given once daily around 10.30 am. Water was available at all times. Cows were milked at 5.30 and 15.30 h daily. The milk yield was determined at each milking and, after leaving the milking parlour, the BWs of the cows were measured automatically.

2.2 Measurement and sample collection

In Period 1, milk was sampled in the morning and afternoon of one day in weeks 0, 2, 4 and 8 and pooled based on the weight of milk produced at the morning and afternoon milking. Milk samples were frozen at −20°C, freeze-dried and kept at −20°C until further analysis. Blood samples (using serum tubes) were drawn from the *Vena jugularis* of each cow between 8.30 a.m. and approximately 10.30 a.m. of one day in weeks 0, 2, 4, 6 and 8 of the trial. On the sampling days, cows had no access to any feed between 5.30 a.m. (start of milking) and the end of sampling procedure. Serum was separated by centrifugation at $2000 \times g$ and 15°C for 20 min and afterwards stored by −20°C. On one morning in weeks 0, 4 and 8, samples of the bile were taken from each cow by percutaneous ultrasound-guided cholecystocentesis [24]. Bile samples were stored at −20°C until further analysis. Milk, serum and bile samples of all cows fed the contaminated diets ($n = 13$) and of 50% of the control cows ($n = 7$) were analysed for DON and DOM-1 at each time of sampling.

In Period 2, milk and blood samples were collected in weeks 16, 18, 20, 22 and 28, and bile samples were taken in weeks 16, 20 and 28 following the same procedure as described for Period 1. Milk, serum and bile samples of all cows fed the contaminated diets ($n = 16$) and of 50% of the control animals ($n = 8$) were analysed for DON and its metabolite at each time of sampling.

In Period 1, samples of the grass- and maize silage were collected twice a week and pooled together over approximately 5 wk. Once weekly concentrates were sampled and pooled over the entire Period 1. Silage samples were dried at 60°C for 72 h. All feed samples were ground to pass through a 1 mm screen for further analysis.

In Period 2, TMR samples were collected four times a week directly after the feeding from each trough and pooled over approximately 4 wk. The sampling procedures for concentrates and silages were the same as described above but the concentrates were pooled approximately every 4 wk. A pooled sample of the used grass silage and maize silage was built when the silo was changed.

In the following depletion period, all cows were fed the control diet with a concentrate proportion of 30% and a mean DON concentration of 0.4 mg/kg DM for the duration of 4 wk. Samples of the morning and afternoon milking were collected twice weekly and pooled to one sample *per* week. The milk samples were frozen at −20°C, freeze-dried

and kept at −20°C until further analysis. After 3 wk of feeding the control ration, blood and milk samples were taken again from each cow following the same procedures as described above.

Sampling procedures for the TMR were similar as described above, but one sample was built for the entire depletion period. Pooled samples of the silages were built correspondingly.

2.3 Analysis

The crude nutrients in feed samples were determined according to the methods of the ‚Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten' (VDLUFA; [25]).

DON in feedstuffs was analysed by HPLC with DAD after clean-up with immunoaffinity columns (IAC) (DONprep®, r-biopharm AG, Darmstadt, Germany) according to Oldenburg *et al*. [26]. β-Glucuronidase is not used in this method. The LOD was 0.03 mg/kg. The mean recovery was 92%.

ZON contamination in feedstuffs was determined with HPLC with fluorescence detection according to a modified VDLUFA method according to Ueberschär [27] described by Dänicke *et al*. [28] with an LOD of 1.1 μg/kg DM. The mean recovery was 86%.

DON and further trichothecene mycotoxins (nivalenol, scirpentriol, T2-tetraol, fusarenon-X, monoacetoxyscirpenol, 15-acetyldeoxynivalenol, neosolaniol, 3-acetyldeoxynivalenol, diacetoxyscirpenol, T2-triol, HT-2 toxin and T-2 toxin) were determined by the Department of Animal Nutrition, Hohenheim University, Stuttgart, in the contaminated and the control triticale according to a GC-MS method [29].

DON and DOM-1 in serum, freeze-dried milk and bile were determined by HPLC with UV detection according to Valenta *et al*. [30] with modifications. Briefly, 1.5 mL serum, 1 mL bile fluid and 2.4 g freeze-dried milk, respectively, were incubated with 6000 U (serum), 8000 U (bile) and 58 000 U (milk) β-Glucuronidase (type H-2, min. 98 800 U/mL, Sigma, Steinheim, Germany) at pH 5.5 (acetate buffer) and 37°C for 16 h (serum and bile) and for 10 h (milk), respectively. Subsequently, serum and bile were extracted with ethyl acetate (bile after pH adjustment to 7) on disposable ChemElut® columns (Varian, Darmstadt, Germany) and cleaned up with IAC (DONtest®, VICAM, Watertown, USA in case of serum and DONprep, r-biopharm AG in case of bile). Freeze-dried milk was extracted with a mixture of ACN and water, defatted with petroleum ether, precleaned with a mixture of charcoal, alumina and celite and cleaned up with IAC (DONprep, r-biopharm AG).

The LODs (S/N greater than 3:1) for DON and DOM-1 were approximately 2 ng/mL in serum, 4 μg/kg in freeze-dried milk (corresponds to 0.5 μg/kg milk with 12.5% DM) and 4 ng/mL in bile. The mean recoveries were 90 and 91% for DON and DOM-1 in serum, both 87% in milk and 95 and 93% in bile.

To confirm the results of the milk analysis, the milk samples of all cows fed contaminated diets and half of the control cows collected in week 28 ($n = 24$) were additionally analysed by LC-ESI-MS/MS. The remainder of the purified sample extracts which were used in HPLC-UV analysis was diluted 1:2 with the HPLC-UV eluent (ACN/water, 13:87 v/v and applied for the LC-MS/MS analysis. The measurements were conducted on an API 4000 QTrap tandem mass spectrometer system (Applied Biosystems, Darmstadt, Germany), conducted to a 1200 series HPLC system of Agilent Technologies (Böblingen, Germany). The LC-ESI-MS/MS method will be described in detail elsewhere (Valenta *et al*., in preparation). Briefly, the separation was carried out on a Betasil Phenyl/Hexyl column (100 mm × 2.1 mm, 3 μm; Thermo Electron Corporation, Runcorn, UK), using a binary gradient of 0.13 mM ammonium acetate in water (pH 7.4, solvent A; described by Klötzel *et al*. [31], and ACN (solvent B). The measurements were performed with multiple reaction monitoring (MRM) in the negative mode, selecting the mass transitions 295 → 265 (DON) and 279 → 249 (DOM-1) for quantification and 295 → 138 (DON) and 279 → 231 (DOM-1) for additional qualifying. Quantification was done by means of calibration curves in the range of 1 – 500 ng/mL for both toxins. The LOD (S/N greater than 3:1) for both DON and DOM-1 in freeze-dried milk was 0.4 μg/kg (corresponds to 0.05 μg/kg milk with 12.5% DM) the mean recovery was 86% (DON) and 89% (DOM-1), respectively.

Results of analysis of mycotoxins and their metabolites were not corrected for recovery.

2.4 Calculations and statistical analysis

Different statistical procedures were applied as residues measured in serum, bile and milk were not normally distributed in the groups fed the mycotoxin-free diets (Control-30, Control-60) whereas the corresponding residues of cows fed the mycotoxin containing diets (Myco-30 and Myco-60) followed the normal distribution. Therefore, in order to compare all four experimental groups statistically, the Kruskal – Wallis median test implanted in the Statistica for Windows™ operating system (Statsoft Inc. 1994) was used.

If data were pooled for the 'concentrate proportion' in Period 2, a normal distribution was achieved and data were analysed using the SAS package (Version 9.1.3, procedure mixed, SAS Institute, Cary, NC, USA). The fixed effect was 'CONC' (concentrate proportion, 1 = 30% concentrate in the diet, 2 = 60% concentrate in the diet). As a regressive component 'INTAKE' (DON intake) and for carry over 'MILK' (milk yield) was considered. We used the ESTIMATE statement to test the differences between the regression coefficients estimated for the dose-dependent

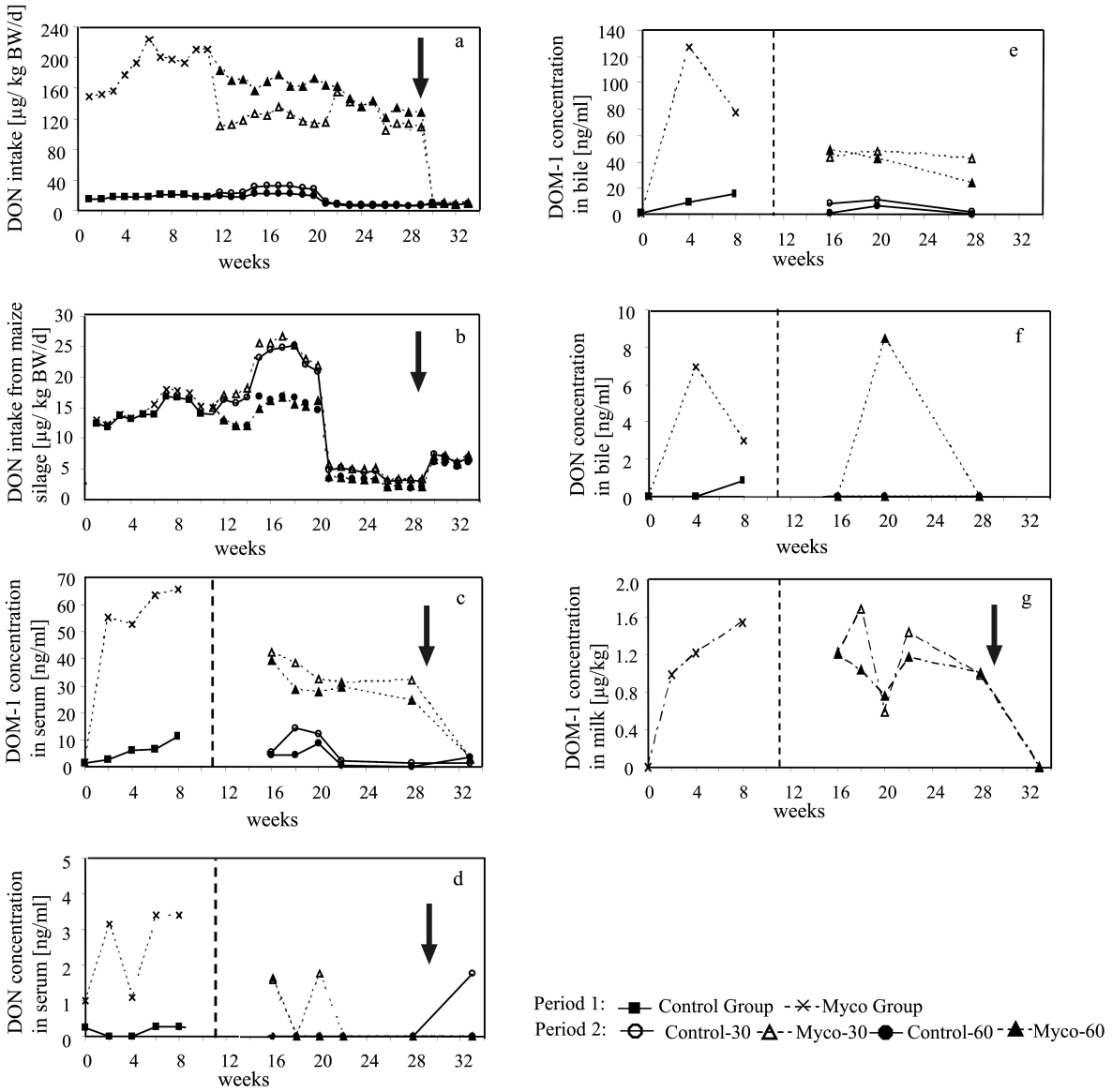

Figure 1. Development of DON intake (a), DON intake from maize silage (b), de-epoxy DON (DOM-1) concentration in serum (c), DON concentration in serum (d), DOM-1 concentration in bile (e), DON concentration in bile (f) and DOM-1 concentration in milk (g) in the feeding groups .
The arrow indicates the beginning of the depletion period (end of feeding the experimental diets).

(INTAKE) and milk yield (MILK)-dependent changes, respectively. By using the REPEATED statement, the individual cow ('Cow') effects resulting from the frequent measurements on the same animal in the course of the experiment were considered.

The following SAS code was used to test the residues in bile and serum (*y*):

PROC MIXED METHOD = REML;
CLASS CONC COW**;**
MODELY = CONC INTAKE (CONC)**/DDFM = KR S;**
LSMEANS CONC/**PDIFF** e adjust = TUKEY
ESTIMATE INTAKE (CONC) 1 -1**;**
REPEATED/TYPE = SP (exp) SUB = COW LOCAL**;**
RUN;

In case of the carry over into milk (*y*), the 'INTAKE' was replaced by 'MILK' in the above model.

DON and DOM-1 concentrations which were lower than the LOD were considered as zero. Only values higher than the LOD were enclosed in the calculation of the metabolite profile and the total carry over rate. In Fig. 1, the estimation

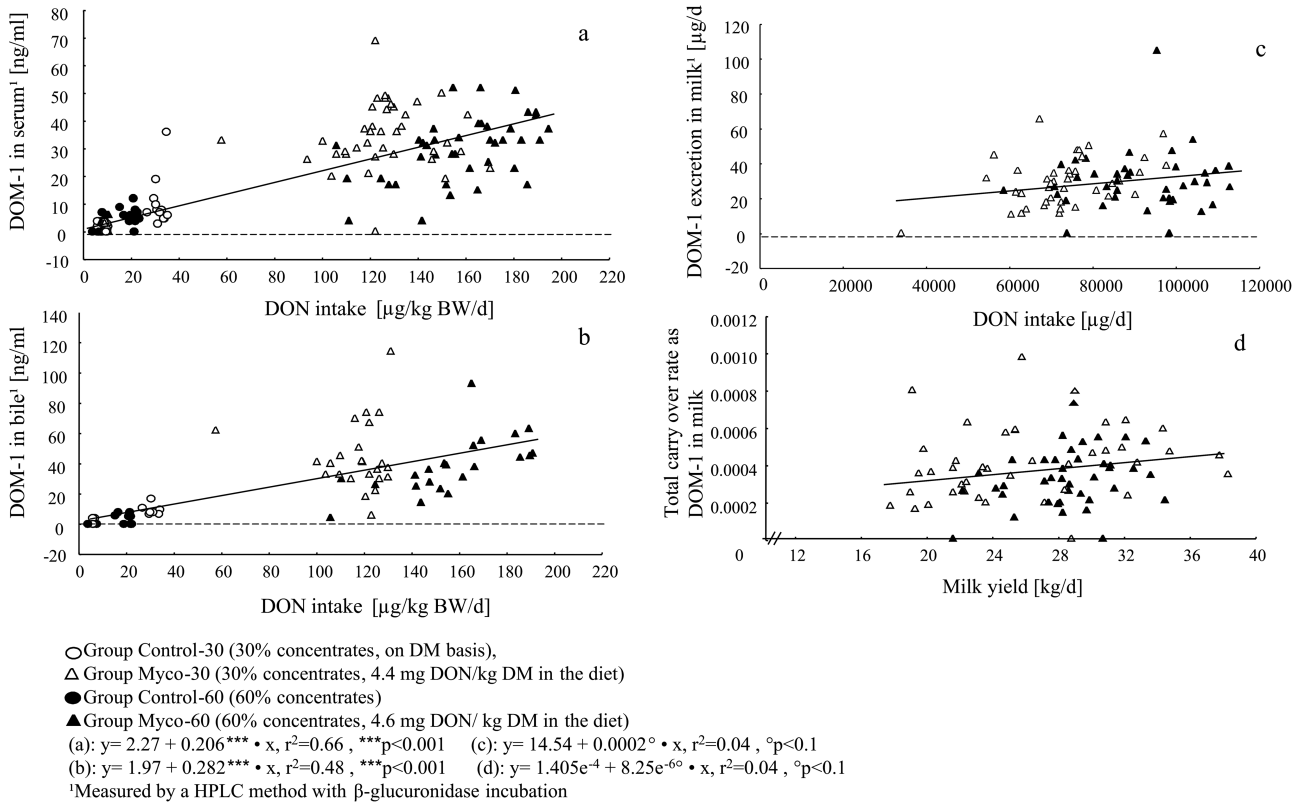

Figure 2. (a, b) Relationship between deoxynivalenol (DON) intake (mg/kg body weight/d) and de-epoxy DON (DOM-1) concentration in (a) serum (ng/ml) and in (b) bile, respectively, in control and mycotoxin groups. (c, d) Relationship between DON intake (µg/d) and DAM-1 excretion in (c) milk (µg/d) and between (d) milk yield (kg/d) and total carry over rate of DON as DOM-1 in milk.

of the arithmetic means included values lower than the LOD with '0'.

DOM-1 concentrations in milk and its excretion in milk were recalculated on the mean original substance of milk (mean DM of milk in this trial: 12.5%).

Differences were considered to be significant at $p < 0.05$, and a tendency was discussed when $p < 0.1$.

Where appropriate, dose-response relationships were considered by a simple linear regression analysis using the Statistica for Windows TM operating system (StatSoft, 1994) (Fig. 2).

Metabolite profile was calculated as following:

$$\text{Metabolite profile} = \frac{\text{DON or de} - \text{epoxy DON concentration in substrate}}{\text{Total DON plus de} - \text{epoxy DON concentration in substrate}} \times 100$$

The carry over rate in milk was estimated as follows:

$$\text{Total carry over rate into milk} = \frac{\text{Total excretion of DON and/or de} - \text{epoxy DON in milk } (\mu\text{g/d})}{\text{Daily DON intake } (\mu\text{/d})}$$

3 Results

In general, for the graphic presentation of the results (Fig. 1), the arithmetic group means were used to get an impression of the time-dependent course (*i.e.* experimental week) of the analysed parameters. For the statistical analyses, we first used the nonparametric Kruskal–Wallis test which is based on the comparison of medians in order to compare all experimental groups. Therefore, Tables 6 and 7 show the medians and the ranges (minimum–maximum) for the analysed parameters. Second, the least square means (LSmeans) and the regression coefficients according to the

Table 3. Composition and mycotoxin concentrations of the concentrates ($n = 3$) and silages used in Period 2 ($n = 4$) (means ± SD)

	Concentrate				Maize silage	Grass silage
	Control-30	Myco-30	Control-60	Myco-60		
Components (%)						
Triticale	50	0	50	25		
Fusarium-contaminated triticale	0	50	0	25		
Soyabean meal	26	26	26.8	26.8		
Maize	20	20	20.8	20.8		
Mineral feed[a)]	1.5	1.5	1.2	1.2		
Calcium carbonate	2.5	2.5	1.2	1.2		
DM (g/kg)	883 ± 18	890 ± 17	884 ± 17	883 ± 11	336 ± 41	291 ± 86
Nutrients (g/kg DM)						
Crude ash	66 ± 3	67 ± 10	52 ± 1	54 ± 1	37 ± 2	93 ± 6
Crude protein	205 ± 8	198 ± 4	203 ± 4	203 ± 4	87 ± 0	123 ± 20
Crude fat	22 ± 6	23 ± 5	23 ± 3	26 ± 5	26 ± 3	24 ± 5
Crude fibre	35 ± 2	35 ± 0	37 ± 2	35 ± 2	188 ± 0	316 ± 13
Acid detergent fibre	47 ± 1	47 ± 1	49 ± 3	47 ± 2	205 ± 2	328 ± 12
Neutral detergent fibre	134 ± 5	120 ± 5	143 ± 23	123 ± 3	396 ± 1	561 ± 14
Mycotoxins						
Deoxynivalenol (mg/kg DM)	0.5 ± 0.4	13.1 ± 2.9	0.3 ± 0.0	7.2 ± 0.7	1.3 ± 1.0	<0.03
ZON (μg/kg DM)	17.3 ± 12.5	146.4 ± 24.3	12.3 ± 2.8	92.4 ± 3.6	85.3 ± 98.1	<1.1

Period 2, week 12 – 29; Control-30, cows fed the control diet with 30% concentrate (on DM basis); Myco-30, cows fed the *Fusarium* toxin-contaminated diet with 30% concentrate (on DM basis); Control-60, cows fed the control diet with 60% concentrate (on DM basis); Myco-60, cows fed the *Fusarium* toxin-contaminated diet with 60% concentrate (on DM basis).

a) *Per* kg mineral feed: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu; 100 mg I; 40 mg Se; 25 mg Co; 1 000 000 IU vitamin A; 100 000 IU vitamin D_3; 1500 mg vitamin E.

Table 4. Composition and mycotoxin concentrations of the TMR used in Period 2 ($n = 5$)

	Group			
	Control-30	Myco-30	Control-60	Myco-60
DM (g/kg)	386 ± 62	394 ± 66	456 ± 59	475 ± 63
Nutrients (g/kg DM)				
Crude ash	68 ± 6	69 ± 6	61 ± 3	66 ± 9
Crude protein	135 ± 12	139 ± 11	165 ± 15	154 ± 15
Crude fat	28 ± 5	28 ± 5	26 ± 4	27 ± 5
Crude fibre	203 ± 10	196 ± 12	142 ± 19	128 ± 53
Acid detergent fibre	220 ± 15	212 ± 16	161 ± 17	169 ± 20
Neutral detergent fibre	391 ± 47	394 ± 41	349 ± 34	349 ± 36
Energy[a)] (MJ/kg DM)				
ME	11.4	11.6	12.4	12.1
NEL	7.0	7.1	7.8	7.5
Mycotoxins[b)]				
Deoxynivalenol (mg/kg DM)	0.6	4.4	0.4	4.6
ZON (μg/kg DM)	35.0	73.8	24.4	72.5

Abbreviations: Period 2, week 12 – 29; Control-30, cows fed the control diet with 30% concentrate (based on DM); Myco-30, cows fed the *Fusarium* toxin-contaminated diet with 30% concentrate (based on DM); Control-60, cows fed the control diet with 60% concentrate (based on DM); Myco-60, cows fed the *Fusarium* toxin-contaminated diet with 60% concentrate (based on D); ME, metabolisable energy; NEL, net energy lactation.

a) Calculation based on nutrient digestibilities measured with wethers [48].

b) Calculation based on analysed concentrations in concentrates and silages.

Table 5. Analyses of *Fusarium* toxins in *Fusarium* toxin-contaminated and in control triticale (measured by a GC-MS method)

	Period 1 Control triticale 1	Period 2 Control triticale 2	Period 1 + 2 Contaminated triticale
Mycotoxins (μg/kg DM)			
Deoxynivalenol	58	238	40 872 (36 980[a)])
ZON	<1.1[b)]	<1.1[b)]	434.7[b)]
Nivalenol	<20	<20	965
Scirpentriol	<5	<5	91
T2-tetraol	<45	<45	<45
Fusarenon-X	<10	<10	<10
Monoacetoxyscirpenol	<2	<2	<2
15-Acetyldeoxynivalenol	<4	<4	522
3-Acetyldeoxynivalenol	<9	<9	204
T2-triol	<6	<6	<6
Neosolaniol	<4	<4	<4
Diacetoxyscirpenol	<6	<6	<6
HT-2 toxin	<2	<2	<2
T-2 toxin	<3	<3	<3

a) Measured by an HPLC method [26].
b) Measured with an HPLC method with fluorescence detection [27].

described SAS procedure were calculated for selected parameters and are presented in Tables 8 and 9 for the pooled effect of concentrate. As the regression coefficients were not significantly different, a pooled graphical presentation was chosen in Fig. 2.

DON and DOM-1 in serum, bile and milk were analysed after treatment with glucuronidase so that the DON and DOM-1 concentrations in these substrates include the sum of unconjugated and conjugated metabolites.

3.1 Triticale

In both periods, we used the same naturally contaminated triticale batch to reach the DON concentration of 4.8 mg/kg (on a reference DM of 88%) in the diet. The *Fusarium* toxin-contaminated triticale contained on average 40.9 mg DON/kg DM (GC-MS method) and 0.4 mg ZON/kg DM, whereas the control triticales contained DON concentrations between 0.06 and 0.2 mg/kg DM (Table 5). The ZON concentrations were below the LOD in both control triticales. The analyses of further mycotoxins revealed 0.97 mg Nivalenol/kg DM, 0.09 mg Scirpentriol/kg DM, 0.52 mg 15-acetyldeoxynivalenol/kg DM and 0.20 mg 3-acetyldeoxynivalenol/kg DM in the contaminated triticale, while the concentration of these *Fusarium* toxins were lower than the LOD in the control triticales (Table 5).

3.2 Mycotoxin intake

3.2.1 Period 1

In the Control Group, the daily DON intake stayed on a nearly constant level (between 15 and 20 μg/kg BW) from week 1 on, whereas, in the Myco Group, the intake increased from approximately 150 μg/kg BW in week 1 to a peak in week 6 (225 μg/kg BW). Afterwards the DON intake decreased to approximately 200 μg/kg BW and stayed at this level (Fig. 1a).

The DON intake originating from the DON contamination in the used maize silage batches is shown in Fig. 1b. Over the entire Period 1, maize silages from three different silos were used in which the DON concentration ranged from 1.7 mg DON/kg DM to 2.1 mg DON/kg DM.

3.2.2 Period 2

In both control groups, the daily DON intake showed a similar course with a slight increase by week 15, with a plateau phase between weeks 15 and 20, and a decrease to a constantly lower level from week 21 on (Fig. 1a). The daily intakes ranged between 6 – 33 μg/kg BW in Group Control-30 and 6 – 23 μg/kg BW in Group Control-60, respectively.

The DON intake in cows in Group Myco-60 had a decreasing tendency over the course of trial and ranged from 121 to 183 μg/kg BW, in Group Myco-30 the intake showed some variations and ranged from 104 to 154 μg/kg BW (Fig. 1a).

In Period 2, the DON concentration in the maize silages from three different silos ranged between 0.3 and 2.4 mg/kg DM. In all groups the highest DON intake from maize silage was reached between weeks 15 and 20, when the maize silage contained 2.4 mg DON/kg DM (Fig. 1b).

3.2.3 Depletion period

In weeks 30 – 34, the used maize silage batch contained 0.7 mg DON/kg DM, which was approximately 50% higher than the maize silage used in weeks 26 – 29. Therefore, the DON intake from maize silage increased after week 29.

Table 6. DON and DOM-1 concentrations in serum, bile and milk of cows in Period 1 (weeks 0–8) (median, range)

Group	Concentration in serum (ng/mL)		Concentration in bile(ng/mL)		Concentration in milk (μg/kg fresh milk)	
	DON	DOM-1	DON	DOM-1	DON	DOM-1
Week 0						
Control	0.0 (0.0–2.0)	0.0 (0.0–7.0)	0.0 (0.0–0.0)	0.0 (0.0–7.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
Myco	0.0 (0.0–4.0)	1.0 (0.0–4.0)	0.0 (0.0–0.0)	0.0 (0.0–6.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
Week 2						
Control	0.0^{B} (0.0–0.0)	3.0^{B} (0.0–5.0)			0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco	2.0^{A} (0.0–12.0)	52.0^{A} (22.0–123.0)			0.0 (0.0–0.0)	1.0^{A} (0.6–1.4)
Week 4						
Control	0.0^{B} (0.0–0.0)	6.0^{B} (5.0–8.0)	0.0^{B} (0.0–0.0)	8.0^{B} (0.0–24.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco	0.0^{A} (0.0–3.0)	54.0^{A} (38.0–70.0)	0.0^{A} (0.0–34.0)	113.0^{A} (10.0–391.0)	0.0 (0.0–0.0)	1.3^{A} (0.9–1.5)
Week 6						
Control	0.0^{B} (0.0–2.0)	7.0^{B} (0.0–11.0)				
Myco	2.0^{A} (0.0–18.0)	70.0^{A} (42.0–79.0)				
Week 8						
Control	0.0^{B} (0.0–2.0)	9.0^{B} (6.0–25.0)	0.0 (0–6.0)	17.0^{B} (0–29.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco	4.0^{A} (0.0–7.0)	67.0^{A} (48.0–79.0)	0.0 (0–9.0)	73.0^{A} (0–175.0)	0.0 (0.0–0.0)	1.5^{A} (1.2–2.2)

Data with various superscript letters are significantly different within a column ($p < 0.05$, Kruskal–Wallis test). Control, cows fed the control diet with 50% concentrate (based on DM); Myco, cows fed the *Fusarium* toxin-contaminated diet with 50% concentrate (based on DM).

3.3 Mycotoxin residues in serum

3.3.1 Period 1

For the interpretation of the results, it has to be taken into account that the time between the last feed intake and the sampling varied from cow to cow as feed was available for *ad libitum* intake and that the time of sampling differed as well (in a range of approximately 2 h). Cows fed the contaminated diet had significantly higher serum concentrations of DON and DOM-1 than cows fed the control diet at all times of sampling during the trial (Table 6). Unmetabolised DON was only detected in concentrations close to the LOD in most cases. The metabolite DOM-1 was detectable in the serum of all cows fed the contaminated diet at all times of sampling. In the Myco Group, the values (min–max) for DON and DOM-1 ranged between below the LOD and 18 ng/mL, and between 22 and 123 ng/mL; while in the Control Group, the values ranged between below the LOD and 2 ng/mL for DON, and below the LOD and 25 ng/mL for DOM-1 (Table 6). Prior to the initial exposure (week 0), DON and its metabolite were sporadically detected in concentrations close to the LOD. In weeks 6 and 8, DON concentrations slightly higher than the LOD were detected in one cow fed the control diet.

The metabolite profile of DON and DOM-1 was not significantly influenced by dietary treatment (data not shown). The time-dependent course is shown in Fig. 1c.

3.3.2 Period 2

Cows fed the *Fusarium* toxin-contaminated diets had significantly higher values of DON and DOM-1 in serum compared to the control animals (Table 7). DOM-1 was detected in the serum of all cows fed the contaminated diet at all times of sampling, except from one Myco cow in week 16. Over the whole Period 2, unmetabolised DON was only detectable four times in four different cows of Group Myco-30 in concentrations between 2 and 14 ng DON/mL, and two times in Group Myco-60 (4 and 9 ng DON/mL). The time-dependent course is shown in Fig. 1d.

DOM-1 concentrations ranged between below the LOD and 69 ng/mL in Group Myco-30 and between 4 and 52 ng/mL in Group Myco-60. Cows fed the low concentrate diets had significantly higher DOM-1 concentrations in serum (Table 8).

Table 7. DON residues in serum, bile and milk of cows in Period 2 and in depletion period (median, range)

	Concentration in serum (ng/mL)		Concentration in bile (ng/mL)		Concentration in milk (μg/kg fresh milk)	
Group	DON	DOM-1	DON	DOM-1	DON	DOM-1
Week 16						
Control-30	0.0 (0.0–0.0)	5.0^{B} (3.0–8.0)	0.0 (0.0–0.0)	8.0^{B} (7.0–10.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-30	0.0 (0–6.0)	48.0^{A} (0–69.0)	0.0 (0.0–0.0)	38.5^{A} (6.0–74.0)	0.0 (0.0–0.0)	1.1^{A} (0.8–2.1)
Control-60	0.0 (0.0–0.0)	4.5^{B} (4.0–5.0)	0.0 (0.0–0.0)	0.0^{B} (0–5.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-60	0.0 (0–9.0)	40.5^{A} (17.0–52.0)	0.0 (0.0–0.0)	45.0^{A} (23.0–93.0)	0.0 (0.0–0.0)	1.1^{A} (0.5–3.2)
Week 18						
Control-30	0.0 (0.0–0.0)	7.5^{B} (6.0–36.0)			0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-30	0.0 (0.0–0.0)	40.0^{A} (26.0–47.0)			0.0 (0.0–0.0)	1.6^{A} (0.8–2.6)
Control-60	0.0 (0.0–0.0)	5.5^{B} (0–7.0)			0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-60	0.0 (0.0–0.0)	33.0^{A} (15.0–38.0)			0.0 (0.0–0.0)	1.1^{A} (0.0–1.8)
Week 20						
Control-30	0.0 (0.0–0.0)	11.0^{A} (7.0–19.0)	0.0 (0.0–0.0)	9.5^{B} (7.0–17.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-30	0.0 (0–14.0)	31.25^{B} (21.0–49.0)	0.0 (0.0–0.0)	38.5^{B} (30.0–114.0)	0.0 (0.0–0.0)	0.6^{A} (0.0–1.2)
Control-60	0.0 (0.0–0.0)	8.5^{A} (6.0–12.0)	0.0 (0.0–0.0)	7.0^{B} (5.0–8.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-60	0.0 (0.0–0.0)	26.5^{B} (13.0–43.0)	0.0 (0–34.0)	42.0^{A} (20.0–63.0)	0.0 (0.0–0.0)	0.7^{A} (0.0–1.3)
Week 22						
Control-30	0.0 (0.0–0.0)	2.0^{B} (0–4.0)			0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-30	0.0 (0.0–0.0)	29.0^{A} (19.0–50.0)			0.0 (0.0–0.0)	1.5^{A} (1.0–1.8)
Control-60	0.0 (0.0–0.0)	0.0^{B} (0–2.0)			0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-60	0.0 (0.0–0.0)	33.0^{A} (4.0–43.0)			0.0 (0.0–0.0)	1.2^{A} (0.9–1.6)
Week 28						
Control-30	0.0 (0.0–0.0)	2.0^{B} (0–2.0)	0.0 (0.0–0.0)	2.0^{B} (0–4.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-30	0.0 (0.0–0.0)	30.5^{A} (20.0–45.0)	0.0 (0.0–0.0)	41.0^{A} (22.0–74.0)	0.0 (0.0–0.0)	1.1^{A} (0.6–1.3)
Control-60	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)	0.0 (0.0–0.0)	0.0^{B} (0.0–0.0)
Myco-60	0.0 (0.0–0.0)	29.0^{A} (4.0–33.0)	0.0 (0.0–0.0)	26.0^{A} (4.0–36.0)	0.0 (0.0–0.0)	1.1^{A} (0.7–1.4)
Week 33 (depletion period[a)])						
Control-30	2.0 (0–3.0)	1.0 (0–4.0)			0.0 (0.0–0.0)	0.0 (0.0–0.0)
Myco-30	0.0 (0.0–0.0)	2.5 (0–6.0)			0.0 (0.0–0.0)	0.0 (0.0–0.0)
Control-60	0.0 (0.0–0.0)	3.0 (0–7.0)			0.0 (0.0–0.0)	0.0 (0.0–0.0)
Myco-60	0.0 (0.0–0.0)	3.0 (0–6.0)			0.0 (0.0–0.0)	0.0 (0.0–0.0)

Data with various superscript letters are significantly different within a column ($p < 0.05$, Kruskal–Wallis test). Concentrations below the LOD were referred as '0;' for abbreviations see Table 6.

a) Depletion period: all cows received the control diet with 30% concentrates (based on DM) after finishing week 29.

DON concentrations in both control groups were below the LOD at any time of sampling while DOM-1 was detectable in concentrations between below the LOD and 36 ng/mL. The concentrations of DOM-1 in serum were significantly linearly correlated with the daily DON intake *per* kilogram BW (Fig. 2a).

Similar to Period 1, *Fusarium* toxin contamination had no effect on the metabolite profile of DON and DOM-1 (data not shown).

3.3.3 Depletion period

After 3 wk of feeding a control diet to all cows, the DOM-1 residues in serum of cows fed the contaminated diets were on the same level as found in the serum of control cows. Unmetabolised DON was found in concentrations close to the LOD in three cows in Group Control-30.

3.4 Mycotoxin residues in bile

3.4.1 Period 1

Before the trial started (week 0) DOM-1 was detected in both groups in concentrations ranging between below the LOD and 7 ng/mL. In week 4, DON and DOM-1 concentrations were significantly higher in the bile of cows in the Myco Group, in week 8 a significant difference only occurred for DOM-1. Bile samples contained DON and DOM-1 concentrations between below the LOD and 34 ng/mL, and below the LOD and 391 ng/mL in the Myco Group, and between below the LOD and 6 ng/mL, and 29 ng/mL, respectively, in the Control Group (Table 6).

In the Control Group the DON and DOM-1 concentration increased slightly over the weeks of the trial while in the Myco Group the concentrations for both DON and DOM-1 reached a peak in week 4 and decreased afterwards (Fig. 1e).

The DOM-1 concentration in bile was significantly linearly correlated with the DOM-1 concentration in serum ($y = 15.78 + 1.33^{**}x$; $r^2 = 0.18$; $^{**}p < 0.01$) if the residues of cows of the control and the mycotoxin fed groups were considered together.

Similar to the results in serum, the metabolite profile in bile remained unaffected by the dietary treatment.

3.4.2 Period 2

Unmetabolised DON was only detected in the bile of one animal in Group Myco-60 in week 20. Cows fed the contaminated diets had significantly higher concentrations of DOM-1 in bile compared to cows fed the control diets. Concentrations of DOM-1 ranged between 6 and 114 ng/mL (Group Myco-30) and between 4 and 93 ng/mL (Group Myco-60). In bile of control cows the values ranged between below the LOD and 17 ng/mL (Table 7). Cows fed the low concentrate diets had significantly higher DOM-1 concentrations in the bile (Table 8).

The DOM-1 concentration in bile was significantly linearly correlated with the DON intake *per* kg BW (Fig. 2b) and with the DOM-1 concentration in serum ($y = 7.583 + 0.891^{***}x$; $r^2 = 0.38$; $^{***}p < 0.001$) if the residues in control and *Fusarium* toxin fed cows are taken together. The metabolite profile was not influenced by *Fusarium* toxin contamination.

Table 8. DOM-1 concentration in serum and bile of all cows in Period 2 (LSmeans ± standard error)

	Concentrate proportion	
	Concentrate-30	Concentrate-60
DOM-1 in serum (ng/mL)	$27.7^{A} \pm 1.5$	$19.3^{B} \pm 1.4$
$\beta_{(\text{DON intake}^{a)} \times \text{DOM-1 in serum})}$	0.24	0.19
DOM-1 in bile (ng/mL)	$35.2^{A} \pm 3.4$	$22.4^{B} \pm 3.4$
$\beta_{(\text{DON intake}^{a)} \times \text{DOM-1 in bile})}$	0.33	0.28

Data with different superscript letters are significantly different within a row ($p < 0.05$). β, Regression coefficient; Period 2, week 12 – 29; concentrate-30, control and myco cows fed 30% concentrates; concentrate-60, control and myco cows fed 60% concentrates.

a) Intake in μg *per* kg BW *per* day.

3.5 Mycotoxin residues in milk

3.5.1 Period 1

Using the HPLC method with β-glucuronidase incubation, no unmetabolised DON could be detected in any milk sample. Concentration of DOM-1 in milk of cows fed the *Fusarium* toxin-contaminated diet ranged between 0.6 and 2.2 μg/kg. Milk samples of control cows did not contain measurable amounts of DOM-1. As shown in Fig. 1 g, the DOM-1 concentration in milk increased until week 8. The daily DOM-1 excretion in milk ranged between 13.3 and 67.6 μg (data not shown). The total carry over rate of ingested DON as DOM-1 into milk ranged between 0.0002 and 0.0006 (Table 6).

3.5.2 Period 2

Unmetabolised DON could not be detected in any sample with the HPLC-UV method. In cows fed the *Fusarium* toxin-contaminated diets DOM-1 was found in concentrations ranging between below the LOD and 3.2 μg/kg milk whereas DOM-1 was not detectable in milk of cows fed the control diets (Table 7).

The time-dependent course revealed a decrease in the DOM-1 concentration in week 20, which was more pronounced in Group Myco-30. Afterwards the concentration stayed on similar levels until the depletion period started (Fig. 1g). Twenty-four milk samples from week 28 were further analysed by using the more sensitive LC-ESI-MS/MS method. Unmetabolised DON was found in only two samples (both in Group Myco-60) in very low concentrations (0.1 μg DON/kg and 0.2 μg DON/kg milk). The HPLC-UV results for DOM-1 were highly significantly

Table 9. DOM-1 concentration in milk of cows fed the *Fusarium* toxin-contaminated diets in Period 2 (LSmeans ± standard error)

	Group	
	Myco-30	Myco-60
DOM-1 concentration in milk (μg/kg)	1.3 ± 0.2	1.0 ± 0.2
$\beta_{(\text{DON intake}^{a)} \times \text{DOM-1 concentration in milk})}$	0.010	0.005
DOM-1 excretion in milk (μg/day)	34.9 ± 3.2	29.1 ± 3.1
$\beta_{(\text{DON intake}^{b)} \times \text{DOM-1 excretion in milk})}$	0.615	0.146
Total carry over rate[c)]	0.0004^{A} ± 0.0	0.0003^{B} ± 0.0
$\beta_{(\text{Milk yield} \times \text{total carry over rate})}$	0.00001	0.00003

Data with different superscript letters are significantly different within a row ($p < 0.05$). β, Regression coefficient; Period 2, week 12–29; Myco-30, cows fed the *Fusarium* toxin-contaminated diet with 30% concentrate; Myco-60, cows fed the *Fusarium* toxin-contaminated diet with 60% concentrate.

a) Intake in μg *per* kg BW *per* day.

b) Intake in μg *per* day.

c) Total excretion of DOM-1 in milk (μg/day) in relation to the daily DON intake (μg/day).

correlated with the LC-ESI-MS/MS results so that the HPLC results were confirmed (for DOM-1: $y_{(\text{LC-ESI-MS/MS})} = 0.01+0.889x_{(\text{HPLC-UV})}$; $^{***}p < 0.001$, $r2 = 0.99$). DOM-1 concentrations determined with the LC-ESI-MS/MS were approximately 11% lower than the corresponding results determined with the HPLC-UV method.

The concentrate proportion neither influenced the DOM-1 concentration in milk nor the DOM-1 excretion in milk but the total carry over rate was significantly higher in cows fed the low concentrate diet (Table 9).

In cows fed the contaminated diets the daily excretion of DOM-1 in milk tended to be linearly correlated with the daily DON intake (Fig. 2c). The DOM-1 concentration in milk was significantly linearly correlated with the DOM-1 concentration in serum ($y = 0.702 + 0.891^{***}$ x, $r^2 = 0.37$, $p < 0.001$).

The total carry over rate of ingested DON as DOM-1 into milk ranged between 0.0002 and 0.001 in Group Myco-30 and between 0.0001 and 0.001 in Group Myco-60 (data not shown). The total carry over rate did not correlate with the DON intake but was significantly linearly correlated with the DOM-1 concentration in the serum of mycotoxin fed cows ($y = 1.946e^{-4} + 5.253e^{-6}{}^{**}x$; $r^2 = 0.11$, $^{**}p < 0.01$). The total carry over rate tended to be linearly correlated with the milk yield (Fig. 2d).

4 Discussion

4.1 DON intake

The present study was conducted to investigate the influence of the concentrate proportion in the ration of dairy cows on the metabolism of DON and its residues in serum, bile and milk after feeding *Fusarium* toxin-contaminated diets over a total period of 29 wk. Naturally contaminated triticale was used to achieve a DON concentration of 5.5 mg/kg DM (corresponds to 4.8 mg/kg on a reference DM of 88%) in both periods. The calculated DON concentration was achieved in Period 1 whereas, in Period 2, the average DON concentrations were approximately 20% lower than calculated although the same triticale was used. This difference probably resulted from the heterogeneity of the used triticale batch and an unequal distribution among contaminated kernels [32–35]. The DON concentration in the diets was calculated by using the analysed values from the maize silage and the concentrates. We analysed TMR samples as well, but DON concentrations in TMR samples were approximately 20% lower than the calculated concentration from the analysed values of the concentrates and silages. This discrepancy can only be explained by the procedure of sampling and an unequal distribution of the *Fusarium* toxins within the pellets. A failure in mixing the TMR is not likely as the nutrient composition of the TMRs did not reveal major deviations from the calculated values.

In the control groups, the DON intake resulted mainly from the background contamination of the used maize silage batches (compare Fig. 1b). The slightly fluctuant DOM-1 concentrations in the serum of the control cows reflected the variability in the DON concentrations in the used maize silages. Furthermore the time-dependent course corresponds to the time-dependent course of the total DM intake (shown at Keese *et al*. [22]).

Compared to Period 1 the DON concentration in the diet fed to the mycotoxin groups in Period 2 were on average 0.7 mg/kg DM (Group Myco-30) and 0.9 mg/kg DM (Group Myco-60) lower than in the ration fed in Period 1. Accordingly the daily DON intakes were lower, which is reflected by lower DON residues in serum and bile in Period 2.

4.2 General aspects of residues in serum, milk and bile

Data about DON residues in physiological substrates and the carry over of DON in milk of dairy cows fed rations with a practically relevant DON concentration are still rare.

In both periods of the present study, and independent of the concentrate proportion, DOM-1 was the predominant component found in the serum samples of cows fed the *Fusarium* toxin-contaminated diets. These results are in accordance with Seeling *et al*. [2] who fed a ration with 60% concentrate and 3.9 mg DON/kg DM to 14 fistulated dairy cows over a period of 4 wk and detected DOM-1 concentrations between 4 and 28 ng/mL in the serum, but no unmetabolised DON. Accordingly Prelusky *et al*. [5] found that less than 1% of DON were systemically absorbed in two cows fed a very high single oral dose of 1.9 mg pure DON/kg BW (920 mg *per* animal). DOM-1 residues were not considered in this study.

In the current study, unmetabolised DON was found in serum in concentrations close to the LOD in Period 1 and occurred only sporadically in Period 2. The DON concentrations in the diets were, with 4.4 (Myco-30) and 4.6 mg/kg DM (Myco-60; Period 2) and 5.3 mg/kg DM (Period 1), respectively, higher than by Seeling *et al*. [2] and this is reflected in higher DON and DOM-1 concentrations in the serum samples. DOM-1 concentrations were significantly linearly correlated with the DON intake (Fig. 2a). Seeling *et al*. [2] detected DOM-1 in serum of cows fed the control diet (0.3 mg DON/kg DM) in concentrations ranging from below the LOD and 5 ng/mL. In the present study, we sporadically found DON concentrations slightly above the LOD, and DOM-1 concentrations up to 7 ng/mL, already before the trial started in individual cows fed the control diets, and up to 25 ng/mL (DOM-1) during the trial, respectively. These residues can be explained by the background contamination coming from the maize silage fed to all experimental groups. Contrasting results were reported by Sabater Vilar [10] who observed a massive average concentration of 32 μg/L of unmetabolised DON in the serum of cows belonging to a dairy herd in The Netherlands with an increased mastitis incidence at the same time. The estimated individual daily DON exposure was, with 4.5 mg, even lower than the background DON concentrations we used in the current study, and those Seeling *et al*. [2] fed to the control animals. Anyway Sabater Vilar [10] concluded that the rumen was not capable of degrading this amount of DON. However, Seeling *et al*. [2] stressed that Sabater Vilar [10] did not reveal any information about the applicability of the applied ELISA method for the detection of DON in serum. Alkaassem *et al*. [36] did neither find a relationship between the occurrence of DON and ZON in physiological substrates and specific symptoms, nor in the course of disease (healing or exitus letalis) in cows treated for *Dislocatio abomasi*.

Metabolism studies with ruminants indicate that DON is completely degraded in the rumen [2, 8, 37]. Urine seems to be the main route of excretion of DON in ruminants; faecal and bilary excretion seems to be less important [2, 6]]. However, it has been shown for pigs that the DON and DOM-1 concentration in bile increases with the DON intake [38]. In the current study, the DOM-1 concentrations in bile were linearly related to the DON intake (Fig. 2b). Unmetabolised DON only occurred sporadically in individual cows fed the *Fusarium* toxin diets, which is in accordance with the corresponding results in serum and milk.

The DON concentration in milk of exposed cows and the carry over of DON in milk has been examined in different studies. Charmely *et al*. [39] neither detected DON nor DOM-1 concentrations above 1 ng/mL in cows with daily DON intakes up to 104 mg by using an HPLC-MS method. Conjugated DON and DOM-1 was not determined. Prelusky *et al*. [5] found only very small amounts of DON (maximum of 4 ng/mL) with and without incubation with glucuronidase after a large single oral dose of 1.7 mg DON/kg BW (this dose was approximately 10- to 13-fold higher than the oral dosage cows consumed in the current study), DOM-1 was not analysed in that study. After three cows were exposed to daily DON intakes between 0.1 and 0.7 mg/kg BW over a period of 5 days, no detectable amounts of the parent toxin were found, but unconjugated DOM-1 was excreted in the milk of all cows over the 5 days period of feeding in concentrations up to 26 ng/mL (conjugated DOM-1 was not analysed) [7]. At 24 h after the last feeding of the contaminated diet unconjugated DOM-1 was no longer detectable in any milk sample [7]. Seeling *et al*. [2] did not detect unmetabolised DON using a HPLC method with β-glucuronidase incubation (LOD: 0.5 μg/kg) while DOM-1 concentrations ranged between 1.6 and 2.7 μg/kg milk in cows with a daily DON intake between 34 up to 76 mg. Using the HPLC-UV method without β-glucuronidase incubation, DOM-1 was only detected in one sample [2] which indicates that DON in milk was mainly present in conjugated form. With a more sensitive GC-MS method (LOD: 0.1 μg/kg), DON concentrations slightly above the LOD were found.

In agreement with Seeling *et al* [2], no unmetabolised DON was detected in the present study using the HPLC-UV method with β-glucuronidase incubation. DOM-1 residues ranged between 0.6 and 2.2 μg/kg (Period 1) and between below the LOD and 3.2 μg/kg milk (Period 2) in cows fed the contaminated diets whereas no DOM-1 residues were detectable in any milk sample of the control cows. It is noticeable that the DOM-1 concentrations in milk were approximately 63% (Myco-30) and 36% (Myco-60) lower in week 20 compared to the results in week 18 (Fig. 1g). This decrease can be traced back to an approximately 25% lower dry matter intake (DMI) the day before the milk samples were taken and a therewith connected decreased DON intake. This decreased DON intake is not visible in Fig. 1a because for the graphic presentation the average weekly DON intake is considered. In a previous study, it was shown that DON is rapidly metabolised to DOM-1 in the rumen and excreted rapidly *via* milk [5]. Any DON residues in the milk of cows dosed with a high single oral dose of 1.7 mg/kg BW were below the LOD after 20 h post-treat-

ment [5] and this dose was approximately 10- to 13-fold higher than the average DON intake in Period 2 of the present experiment. However, this decrease in the DOM-1 concentration of milk samples in week 20 was more pronounced in the Group Myco-30, and the reasons for that remained unclear. The DOM-1 concentrations in serum were not noticeably lower compared to other weeks as serum samples were drawn a day after sampling for milk, and the DMI was already at a normal level.

The carry over rate of DON as DOM-1 was almost negligible with values ranging between 0.0002 – 0.0006 in Period 1 and 0.0002 – 0.001 (Myco-30) and 0.0001 – 0.0011 (Myco-60), respectively, in Period 2. Seeling *et al*. [2] reported about slightly higher carry over rates of DON as DOM-1, with values ranging between 0.0004 and 0.0024. Possible explanations could be a distinctly shorter experimental period (28 days) of the study by Seeling *et al*. [2], and a different bioavailability of DON from the main DON source used (wheat *vs*. triticale). In both studies the carry over was not linearly correlated with the DON intake. In the current experiment the carry over tended to be higher with an increased milk yield, but not as pronounced as reported by Seeling *et al*. [2]. Seeling *et al*. [2] used 11 cows with milk yields between 9.6 and 42.7 kg/day for their carry over studies, whereas in the present experiment the milk yield was in a considerably smaller range with daily milk yields between 18.0 and 38.0 kg/day. That could be the reason why we did not observe a more pronounced relationship between milk yields and carry over rate. As a reason for this increase in the total carry over rate with increased milk yields, Seeling *et al*. [2] supposed that there might be a passive concentration-dependent permeability from blood to alveolar cells of the mammary gland for DON and DOM-1, which was suggested for aflatoxin M_1 as well [40]. Another factor influencing the blood-udder-barrier and therewith connected to the carry over rate might be the udder condition itself as udder infections can increase the permeability of the blood-udder-barrier [40]. During the 29 wk of the current trial we observed some cases of clinical mastitis in individual cows but we could not detect an obvious relation between the occurrence of clinical signs of an udder infection and elevated DOM-1 concentrations in milk samples of the affected cows. Anyway it has to be stressed that cows showing clinical signs of mastitis were treated immediately and that the determination of the somatic cell count was just adequate to get an impression about the udder health of the animals in this study for the reasons discussed by Keese *et al*. [22].

After 1 wk of feeding a control diet to all cows (depletion period) the milk samples neither contained any DON nor DOM-1 concentrations above the LOD. This result was expected as in the experiment by Prelusky *et al*. [5] no measurable DON residues occurred 20 h post-treatment after dosing cows with an approximately 10- to 13-fold higher dose than we fed in Period 2.

4.3 Specific aspects of the concentrate effects

Our hypothesis was that cows fed diets with a high concentrate proportion of 60% may be in a status of SARA and that SARA-induced changes in the ruminal environment and/or in the ruminal mucosa may lead to an altered metabolism of DON and/or absorption of the unmetabolised parent toxin across the ruminal epithelium in the blood circulation. This hypothesis was not confirmed in the present experiment as we observed neither an altered metabolite profile of DON and DOM-1, nor practically relevant amounts of unmetabolised DON in serum and milk in cows fed 50 and 60% concentrates. Duffield *et al*. [41] defined pH values of 5.9 and 6.2 as critical threshold of developing SARA for oro-ruminal probe samples. As shown by Keese *et al*. [23] critical pH values occurred only four times in the Myco Group over the whole Period 1, in Period 2 the minimum observed pH values in cows fed on a high concentrate level were all higher than 6.3, so that no critical pH values occurred which would indicate the presence of SARA. However, a temporary exceeding of the threshold values cannot be ruled out due to the technique of sampling for ruminal fluid (compare Keese *et al*. [23]) but the barrier function of the ruminal mucosa seemed to remain effective as no relevant amounts of unmetabolised DON were detectable in serum and milk.

Unexpectedly, cows fed the low concentrate diets in Period 2 had significantly higher DOM-1 concentrations in serum and bile and a significantly higher total carry over rate compared to cows fed the high concentrate diets. These higher residues are not explainable by a higher DON intake in Group Myco-30, as they consumed approximately 20% less DON *per* kg BW *per* day compared to Group Myco-60 due to a lower DMI. The main difference between the ration of the low and the high concentrate groups was – beside the concentrate proportion – the proportion of maize- and grass silage. As the used grass silage batches were virtually free of DON the only other DON source – beside the contaminated triticale – was the maize silage proportion of the ration which amounted to 20% in Group Myco-60 and 35% in Group Myco-30. A possible explanation might be that the release and bioavailability of DON coming from the maize silage might be different from the kinetics from the contaminated triticale but to date there is no data available about the bioavailability of DON in cows depending on the substrate. Blood samples were taken between 8:30 and 11:30 a.m. in the morning and cows had no access to food from 5:30 a.m., when the milking started, onwards to the finish of sampling. As fibre compounds are degraded more slowly than carbohydrates [42, 43], it could be possible that DON is released more slowly from maize silage and would consequently be detectable longer in the serum. Another reason might be the underestimation of the true DON concentration in the diet by the presence of conjugated DON metabolites, the so-called masked mycotox-

ins, which are produced in plants as a mechanism to reduce the toxicity of mycotoxins [44]. Berthiller *et al*. [45] found a DON-3-β-D-glucopyranoside (D3G) in naturally contaminated *Fusarium*-infected wheat and maize samples ranging from 4 to 12% of the DON concentration. This conjugated metabolite cannot be detected with the applied HPLC-UV method. During digestion, masked mycotoxins might be hydrolysed, releasing the free toxin. This metabolism was shown for ZON-4-β-D-glucopyranoside [46]. Up to now, however, there is no data available about the bioavailability and stability of D3G in the gastrointestinal tract of ruminants.

It can be concluded that no significant amounts of unmetabolised DON can pass the ruminal epithelium, neither if lactating dairy cows were fed a ration with 50% concentrate proportion and 5.3 mg DON/kg DM, nor if a ration with 60% concentrate and 4.6 mg/kg DM was fed for a total period of 29 wk. Furthermore the metabolism of DON was not influenced by the concentrate proportion in the diet. No measurable amounts of the parent toxin were found in the milk using an HPLC-UV method after β-glucuronidase incubation, and only trace amounts ($\leq$0.2 μg/kg) in 2 of 24 milk samples analysed with LC-MS/MS. The carry over of the less toxic metabolite DOM-1 into milk can be regarded as negligible. In contrast to our hypothesis, cows fed 30% concentrate showed higher DOM-1 concentrations in serum and bile, probably as a result of an underestimation of the DON concentration in their diet and/or an altered bioavailability of DON due to a higher proportion of maize silage in the feed of this group. The metabolism and absorption of DON under different ruminal conditions (*e.g*. ruminal acidosis, disease-related alterations in the ruminal mucosa) needs to be further investigated.

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