

## Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in pigs



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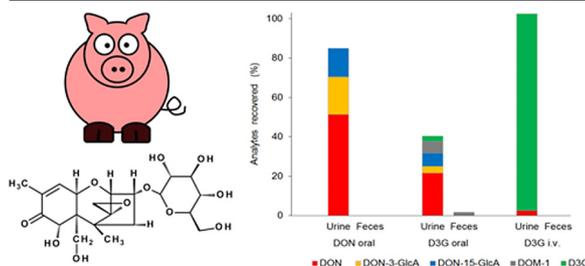
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### HIGHLIGHTS

- The metabolism of deoxynivalenol-3-glucoside (D3G) was elucidated in pigs.
- D3G (oral) was nearly completely hydrolyzed in pigs, but only partially absorbed.
- Two isomers of deoxynivalenol (DON) glucuronides were detected in pig urine.
- Two times less urinary metabolites were excreted for D3G compared to DON.
- D3G (i.v.) was almost exclusively excreted in unmetabolized form *via* urine.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Plants can metabolize the *Fusarium* mycotoxin deoxynivalenol (DON) by forming the masked mycotoxin deoxynivalenol-3-β-D-glucoside (D3G). D3G might be cleaved during digestion, thus increasing the total DON burden of an individual. Due to a lack of *in vivo* data, D3G has not been included in the various regulatory limits established for DON so far. The aim of our study was to contribute to the risk assessment of D3G by determination of its metabolism in pigs. Four piglets received water, D3G (116 μg/kg b.w.) and the equimolar amount of DON (75 μg/kg b.w.) by gavage on day 1, 5 and 9 of the experiment, respectively. Additionally, 15.5 μg D3G/kg b.w. were administered intravenously on day 13. Urine and feces were collected for 24h and analyzed for DON, D3G, deoxynivalenol-3-glucuronide (DON-3-GlcA),

**Abbreviations:** 3-ADON, 3-acetyl-deoxynivalenol; 15-ADON, 15-acetyl-deoxynivalenol; b.w., body weight; D3G, deoxynivalenol-3-β-D-glucoside; DON, deoxynivalenol; DON-3-GlcA, deoxynivalenol-3-glucuronide; DON-15-GlcA, deoxynivalenol-15-glucuronide; DON-GlcA, deoxynivalenol-glucuronide; DOM-1, de-epoxy deoxynivalenol; DOM-1-GlcA, DOM-1-glucuronide; JECFA, Joint FAO/WHO Expert Committee on Food Additives; MeOH, methanol; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PBS, phosphate buffered saline;  $R_A$ , apparent recovery;  $R_E$ , recovery of the extraction step; SSE, signal suppression/enhancement; UHPLC, ultra high performance liquid chromatography.

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Swine  
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deoxynivalenol-15-GlcA (DON-15-GlcA) and deepoxy-deoxynivalenol (DOM-1) by UHPLC–MS/MS. After oral application of DON and D3G, in total  $84.8 \pm 9.7\%$  and  $40.3 \pm 8.5\%$  of the given dose were detected in urine, respectively. The majority of orally administered D3G was excreted in form of DON, DON-15-GlcA, DOM-1 and DON-3-GlcA, while urinary D3G accounted for only  $2.6 \pm 1.4\%$ . In feces, just trace amounts of metabolites were found. Intravenously administered D3G was almost exclusively excreted in unmetabolized form *via* urine. Data indicate that D3G is nearly completely hydrolyzed in the intestinal tract of pigs, while the toxin seems to be rather stable after systemic absorption. Compared to DON, the oral bioavailability of D3G and its metabolites seems to be reduced by a factor of up to 2, approximately.  
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## 1. Introduction

Mycotoxins, secondary metabolites of different molds, are frequent contaminants of food and feed and cause various adverse health effects in plants, animals and humans. Deoxynivalenol (DON), mainly produced by *Fusarium graminearum* and *Fusarium culmorum*, is one of the economically most relevant mycotoxins worldwide (CAST, 2003). DON exhibits its biological activity by binding to the 60S subunit of eukaryotic ribosomes with subsequent inhibition of the protein biosynthesis (summarized by Arunachalam and Doohan, 2013). In animals, DON induces clinical symptoms like anorexia, emesis, weight loss or immunomodulation (reviewed by Pestka, 2007), while the mycotoxin is associated with outbreaks of gastroenteritis in humans (summarized by Pestka, 2010). As a consequence, regulatory limits in cereal-based food have been established for DON e.g., by the European Commission (European Commission, 2006).

In contrast, deoxynivalenol-3- $\beta$ -D-glucoside (D3G) has not been included in these regulations so far. This masked mycotoxin is formed in plants by enzymatic conjugation of glucose to DON (Poppenberger et al., 2003), thus representing an important defense mechanism of plants against *Fusarium* related diseases (Lemmens et al., 2005). D3G can be found in different cereal crops (Berthiller et al., 2005; Lancova et al., 2008), as well as in animal feed and foodstuff (De Boevre et al., 2012; Malachova et al., 2011). In grains, the molar D3G/DON ratio varies strongly in dependence on factors like genotype or season, but typically reaches levels of around 20% (summarized by Berthiller et al., 2013). However, this ratio can further increase during food processing, resulting in D3G concentrations even exceeding those of DON (Varga et al., 2013).

Despite its frequent occurrence, toxicological data regarding D3G are still rare. Poppenberger et al. (2003) showed that D3G inhibits protein biosynthesis to a far lower extent than its parent toxin in wheat ribosomes. Nevertheless, there are major concerns that ingested D3G might be hydrolyzed in the intestinal tract of mammals, thus releasing its aglycon and increasing the total DON load of an individual (Joint FAO/WHO Expert Committee on Food Additives, JECFA, 2011). Although the toxin seems to be rather stable under conditions simulating early stages of the mammalian digestion (Berthiller et al., 2011; De Nijs et al., 2012), certain lactic acid bacteria were capable to cleave D3G *in vitro* (Berthiller et al., 2011). Furthermore, incubation of human fecal samples with D3G resulted in an extensive liberation of DON (Dall'Erta et al., 2013; Gratz et al., 2013). In 2012, the first *in vivo* reports on the fate of orally administered D3G in rodents became available (Nagl et al., 2012; Veršilovskis et al., 2012). Our group demonstrated that D3G is hydrolyzed in the digestive tract of rats. The liberated DON was further metabolized, but only poorly absorbed. We therefore concluded that D3G is of considerably lower toxicological relevance than DON in rats (Nagl et al., 2012). However, for risk analysis of D3G more data regarding the toxicity and metabolism of this masked mycotoxin are urgently needed (JECFA, 2011).

The situation is quite different for DON, whose toxic effects have been investigated for decades. Notably, major differences in the

susceptibility to DON among animal species were determined, which are most likely related to species-dependent variations in the metabolism of DON (summarized by Pestka, 2007). In principle, ingested DON can be either de-epoxidated by anaerobic bacteria or conjugated to glucuronic acid by mammalian UDP-glucuronosyltransferases (UGTs). While the formation of de-epoxy deoxynivalenol (DOM-1) by ruminal or intestinal microbiota occurs prior to systemic absorption, glucuronidation of DON has so far only been described for liver microsomes (reviewed by Maresca, 2013). Both metabolites, DOM-1 as well as DON-glucuronide (DON-GlcA) are less cytotoxic than their parent toxin (Sundstøl Eriksen et al., 2004; Wu et al., 2007). In recent years, highly sophisticated analytical methods elucidated the metabolic pattern of DON in more detail. For example, the species-specific formation of different DON-GlcA isomers, namely DON-3-glucuronide (DON-3-GlcA), DON-15-glucuronide (DON-15-GlcA), DON-7-glucuronide and DON-8-glucuronide has been proposed (Maul et al., 2012; Uhlig et al., 2013; Warth et al., 2013). Furthermore, the occurrence of novel DON metabolites has been revealed in rats and chickens (Wan et al., 2014). Generally, pigs are considered to be the species most sensitive to DON exposure. This might be attributed to the species' poor ability to detoxify DON to DOM-1 and the high absorption rate of unmetabolized DON (Pestka, 2007). As these characteristics are very similar to what is currently known about human DON metabolism (reviewed by Turner et al., 2012), swine can be regarded as most suitable animal model in this research field.

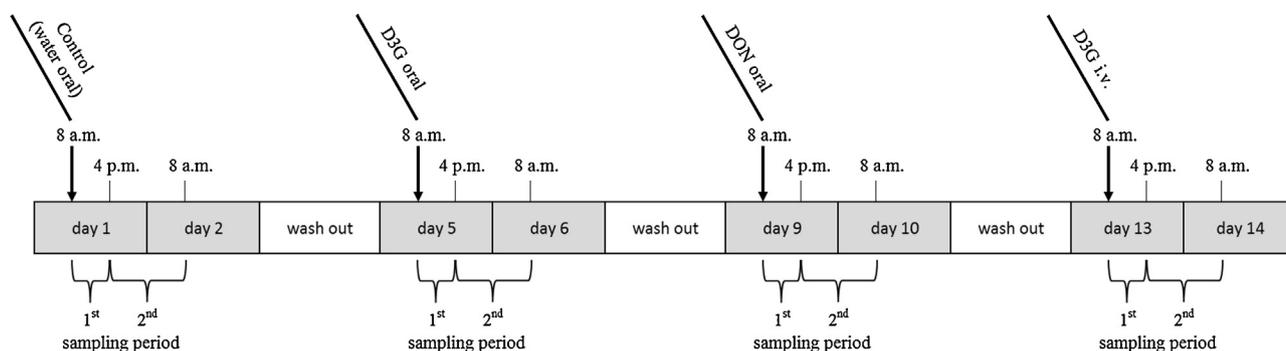
The aim of our study was to elucidate whether D3G has the potential to be reactivated *in vivo* by investigation of its metabolism in pigs. For this purpose, piglets were orally and intravenously dosed with D3G and their excreta subsequently analyzed for DON, D3G, DON-3-GlcA, DON-15-GlcA and DOM-1 by a validated LC–MS/MS based biomarker method. The implementation of an additional oral DON treatment allowed a comparison between absorption rates of D3G and its parent toxin. This study significantly extends the current knowledge about D3G metabolism *in vivo* and therefore facilitates an estimation of the potential health risk of this masked mycotoxin.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Methanol (MeOH, LC grade) and glacial acetic acid (p.a.) were obtained from VWR International GmbH (Vienna, Austria), while ammonium acetate (MS grade) and  $\beta$ -glucuronidase (*Escherichia coli*, Type IX-A) were purchased from Sigma–Aldrich (Vienna, Austria). Reagents for preparation of phosphate buffered saline (PBS; sodium chloride, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate) were from Merck GmbH (Vienna, Austria). Water was purified using a Purelab Ultra system (ELGA LabWater, Celle, Germany).

DON and DOM-1 standards were purchased from Romer Labs GmbH (Tulln, Austria). D3G was either purified from DON treated



**Fig. 1.** Experimental design. After an acclimatization period of five days, four animals received water, D3G and DON by gavage on day 1, 5, and 9, respectively. On day 13, D3G was administered intravenously. After each of the treatments, animals were separated and urine and feces were collected in two different time periods (0–8 h, 8–24 h after administration).

wheat as previously described (Berthiller et al., 2005), or generated by *in vitro* enzymatic synthesis and preparative HPLC purification (manuscript in preparation). DON-3-GlcA was produced by chemical synthesis (Fruhmann et al., 2012). A combined multi standard stock solution, containing 50  $\mu\text{g}/\text{mL}$  DON, D3G, DON-3-GlcA and DOM-1, was prepared in MeOH and stored at  $-20^\circ\text{C}$ . Further dilutions for spiking experiments and calibrants were prepared in MeOH/water (20/80, v/v).

## 2.2. Animals and study design

Male crossbred piglets (sow: Landrace  $\times$  Large White, boar: Pietrain; 28 days old,  $7.1 \pm 0.9$  kg, non-castrated) were obtained from a commercial swine producer and allowed to acclimatize for five days. Animals were housed pairwise in metabolic cages and had free access to water. Feed was provided restrictively twice per day (5% of body weight/day) to ensure fast uptake of the diet. Before the start of the experiment, the diet was tested for its concentrations of DON, D3G and acetylated forms of DON (3-acetyl-deoxynivalenol, 3-ADON; 15-acetyl-deoxynivalenol, 15-ADON).

Using a repeated measures design, the piglets ( $n=4$ ) received water (negative control), D3G (116  $\mu\text{g}/\text{kg}$  b.w.) and the equimolar dose (0.25  $\mu\text{mol}/\text{kg}$  b.w.) of DON (75  $\mu\text{g}/\text{kg}$  b.w.) per gavage on day 1, 5 and 9 of the experiment, respectively. In addition, D3G was administered intravenously (15.5  $\mu\text{g}/\text{kg}$  b.w., 0.03  $\mu\text{mol}/\text{kg}$  b.w.) on day 13 (Fig. 1). Stock solutions of D3G (1.2 mg/mL) and DON (1 mg/mL) were prepared by dissolving the solid standards in water (oral application) or physiological saline (0.9% NaCl, Fresenius Kabi, Graz, Austria; intravenous application). Volumes between 700  $\mu\text{L}$  and 1000  $\mu\text{L}$  were administered orally using polyvinyl chloride catheters (diameter 4.7 mm, length adapted to size of piglets, Medinorm, Spießen, Germany). Intravenous administration was performed by cannulation of the ear vein with sterile catheters (0.9  $\times$  25 mm, VasoVet distributed by B. Braun, Melsungen, Germany). After each of the treatments, animals were separated and urine and feces of individual piglets were collected for 0–8 h and 8–24 h after dosing. Samples were frozen and stored at  $-20^\circ\text{C}$  until further analysis.

The animal experiment was approved by the Institutional Ethics Committee and the national authority according to §8ff of the Austrian Law for Animal Experiments, Tierversuchsgesetz – TVG (GZ 68.205/0236-II/3b/2012).

## 2.3. Sample preparation

For preparation of urine samples, a dilute-and-shoot approach was used. After centrifugation (10 min,  $14,000 \times g$ ), 20  $\mu\text{L}$  of urine samples were added to 180  $\mu\text{L}$  of MeOH/water (20/80, v/v) and

vortexed for 15 s. Thereof, 5  $\mu\text{L}$  were injected into the LC–MS/MS system. In addition, some samples were enzymatically hydrolyzed to investigate the presence of DOM-1-glucuronide (DOM-1-GlcA). To this end, 180  $\mu\text{L}$  of PBS (75 mM, pH 7.4) containing 9000 units of  $\beta$ -glucuronidase were mixed with 20  $\mu\text{L}$  of centrifuged urine samples. After incubation for 18 h at  $37^\circ\text{C}$ , digested samples were clarified by centrifugation (10 min,  $14,000 \times g$ ) for LC–MS/MS analysis.

Clean-up of feces samples was performed according to Nagl et al. (2012). In brief, 250 mg of freeze-dried and homogenized samples were extracted three times (40/40/20 min) with MeOH/water (50/50, v/v, 3/2/2 mL). Following protein precipitation with cold MeOH, aliquots were evaporated and reconstituted in MeOH/water (20/80, v/v, corresponding to a final sample to solvent ratio of 1:56). Afterwards, samples were centrifuged (10 min,  $14,000 \times g$ ) and 5  $\mu\text{L}$  of the supernatant were injected into the LC–MS/MS system.

## 2.4. UHPLC–MS/MS parameters

Analyses were carried out on a 1290 Infinity series UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a 4000 QTrap mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo V electrospray ionization (ESI) source. Chromatographic separation was carried out on a Zorbax RRHD Eclipse XDB-C18 column (2.1 mm  $\times$  150 mm, 1.8  $\mu\text{m}$ , Agilent Technologies, Waldbronn, Germany) equipped with a C<sub>18</sub> security guard cartridge (Phenomenex, Aschaffenburg, Germany). Eluent A consisted of water and eluent B of MeOH/water (97/3, v/v), both containing 0.01% acetic acid and 5 mM ammonium acetate. After an initial period of 2 min at 5% B, the proportion of B was increased linearly to 100% (reached at 6.4 min), followed by a hold time of 2 min at 100% B. Afterwards, the column was re-equilibrated for 2 min at 5% B. The temperature of the column oven was set to  $35^\circ\text{C}$ , while 5  $\mu\text{L}$  of sample volume were injected into a flow of 250  $\mu\text{L}/\text{min}$ . Analytes eluted in the order of DON-3-GlcA (4.9 min), D3G (5.7 min), DON (5.9 min) and DOM-1 (6.3 min). Mass spectrometric detection was performed using negative ESI and selective reaction monitoring mode as described by Nagl et al. (2012).

## 2.5. Method validation and data evaluation

The determined method performance parameters include apparent recovery ( $R_A$ ), signal suppression/enhancement (SSE), recovery of the extraction step ( $R_E$ ), repeatability (RSD), as well as limits of detection (LODs) and quantification (LODs). Prior and after extraction, blank urine and feces samples (collected from piglets after negative control treatment) were spiked with standard compounds in triplicates at 5 and 4 spiking levels, respectively. The

**Table 1**  
Method performance parameters for the pig urine ( $n=15$ ) and feces ( $n=12$ ).

Matrix	Analyte	$R_A^a \pm RSD$ (%)	$SSE^b \pm RSD$ (%)	$R_E^c \pm RSD$ (%)	LOD <sup>d</sup> (ng/ mL)	LOQ <sup>d</sup> (ng/ mL)
Urine	DON	56 ± 1	56 ± 9	102 ± 9	0.9	8.0
	D3G	62 ± 9	62 ± 4	100 ± 5	1.3	2.1
	DON-3-GlcA	114 ± 3	122 ± 8	94 ± 10	9.0	37.3
	DOM-1	73 ± 2	72 ± 10	103 ± 8	1.4	3.7
Feces	DON	54 ± 10	63 ± 5	85 ± 9	3.4	11.2
	D3G	52 ± 10	62 ± 14	95 ± 12	2.6	8.7
	DOM-1	59 ± 9	59 ± 4	89 ± 6	3.2	10.8

<sup>a</sup> Apparent recovery.

<sup>b</sup> Signal suppression/enhancement.

<sup>c</sup> Extraction recovery.

<sup>d</sup> Limits of detection and quantification in spiked measurement solutions.

method for determination of DON, D3G, DON-3-GlcA and DOM-1 in urine was validated in a working range of 3–100 ng/mL in the measurement solution. For feces, method performance characteristics were determined for DON, DOM-1 and D3G in a working range between 30 and 300 ng/mL. Data evaluation was performed as stated in Nagl et al. (2012). Since oral DON and D3G administrations were incomplete in one of the piglets, data from the respective animal were not included in the calculations.

### 3. Results and discussion

#### 3.1. Sample preparation and method performance

Interfering matrix components can represent a major challenge in analysis of DON and its metabolites in biological samples, especially in urine (Gambacorta et al., 2013; Warth et al., 2011). During method development, different sample clean-up techniques, including solid phase extraction, salting-out assisted liquid–liquid extraction and dilute-and-shoot (DAS), were tested. Since DAS comprised satisfactory apparent recoveries of analytes

(56–114%) and simple sample preparation, this approach was chosen for final analysis of urine samples. Due to the dilution of samples by a factor of 10, overall limits of detection (LODs,  $S/N=3$ ) for DON, D3G, DON-3-GlcA and DOM-1 were 9, 13, 90 and 14 ng/mL, respectively. Corresponding limits of quantification (LOQs,  $S/N=10$ ) of 80, 21, 373 and 37 ng/mL were obtained. In standard solutions, LODs and LOQs ranged between 0.5–3.3 and 1.5–9.4 ng/mL. Detailed results of method validation are provided in Table 1.

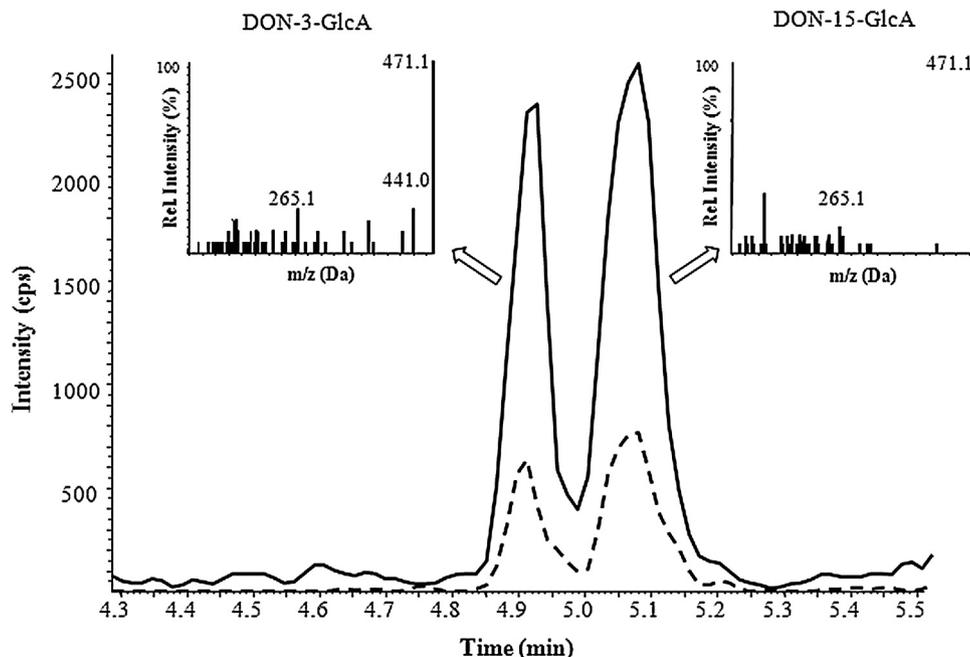
Besides DON-3-GlcA, a second DON-GlcA peak was detected in contaminated urine samples (Fig. 2). Further investigations of this peak by MS/MS experiments and enzymatic treatment according to Warth et al. (2012) suggested the presence of DON-15-GlcA. Most prominently, a fragment of  $m/z$  441 (resulting from the loss of  $CH_2O$  from C15) is observed in DON-3-GlcA, but not in DON-15-GlcA (see inlays of Fig. 2). Due to the lack of a suitable standard, quantification of DON-15-GlcA was performed using DON-3-GlcA calibrants. The comparatively higher MS response of DON-15-GlcA reported by Warth et al. (2012) was verified with our instrumental setup and taken into consideration for all further calculations (correction factor 1.88).

For feces, sample preparation consisted of a three step extraction, followed by protein precipitation. Reduced apparent recoveries (54–59%) were caused by matrix suppression rather than by insufficient extraction of analytes (Table 1). Dilutions of the samples by a factor of 56 resulted in LODs of 188, 146, 179 ng/g for DON, D3G and DOM-1, respectively, while corresponding LOQs of 626, 486 and 605 ng/g were determined.

To sum up, matrix interferences could be observed for both matrices and all analytes. Although their extent was reduced to a level acceptable for analysis of our samples, the importance of a validation for each analyte–matrix combination was once more highlighted.

#### 3.2. Clinical symptoms

DON can cause severe adverse health effects in pigs, e.g., anorexia, emesis or weight loss (reviewed by Pestka, 2007). Due to



**Fig. 2.** SRM chromatogram ( $-m/z$  471  $\rightarrow$  113,  $---$   $m/z$  471  $\rightarrow$  265) of DON-3-GlcA and DON-15-GlcA in a urine sample of a piglet orally dosed with DON. In enhanced product ion scans (inlays, collision energy =  $-40$  eV) the fragmentation product of  $m/z$  471  $\rightarrow$  441 is absent in the second DON-GlcA-isomer, suggesting the presence of DON-15-GlcA.

animal welfare considerations as well as possible influences on the toxins' oral bioavailability (in case of emesis), amounts of administered toxins were chosen on the basis of no observed adverse effect levels for oral (Forsyth et al., 1977), and intravenous DON application (Prelusky, 1993; Prelusky and Trenholm, 1993) in pigs, respectively. Independent of the treatment, neither retching nor emesis was observed in any of the animals. Feed intake was noticed for all piglets within 30 min after toxin administrations.

### 3.3. Detected amounts of excreted toxins in urine and feces

In the individual sampling periods, volumes of collected urine ranged between 22 and 838 mL per piglet, while amounts of freeze-dried feces varied between 2 and 22 g. In urine, concentrations of DON, D3G, DON-3-GlcA, DON-15-GlcA and DOM-1 were between 105 and 1420 ng/mL, 50.6 and 1100 ng/mL, 542 and 1030 ng/mL, 198 and 705 ng/mL and 84.8 and 501 ng/mL, respectively. Of all the analytes tested only DOM-1 was found in feces, with concentrations ranging between 914 and 1220 ng/g. To facilitate comparability between the excreted amounts of different toxins, results are expressed as molar amounts henceforth.

#### 3.3.1. Negative control (water administration)

After oral application of water, traces of DON could be determined in urine samples of the first sampling period (Table 2). The provided feed was analyzed for DON, D3G, 3-ADON and 15-ADON before the start of the experiment and additional feed samples were taken on each of the treatment days. LC-MS/MS measurements revealed low concentrations of DON ( $67 \pm 28 \mu\text{g/kg}$ ) in the diet, whereas none of the other analytes could be quantified (LOQs  $20 \mu\text{g/kg}$ ). Thus, the minimal DON contamination of the diet already led to detectable DON concentrations in urine. However, the daily amount of DON ingested *via* feed reflected only  $\leq 2\%$  of the toxin doses administered in the following treatments and was therefore not considered in further calculations.

#### 3.3.2. Oral DON administration

Following oral administration of DON, we found DON, DON-3-GlcA and DON-15-GlcA in urine of piglets. The majority of analytes was excreted in the first sampling period (Table 2), thus confirming a fast elimination of the mycotoxin (Pestka, 2007). DON was the major urinary metabolite, representing  $62.1 \pm 6.2\%$  of the total analytes excreted in urine. The percentage of

glucuronidated DON in pig urine ( $37.9 \pm 6.2\%$ ) corresponds to values obtained in previous studies (Dänicke et al., 2005; Sundstøl Eriksen et al., 2003), in which DON-GlcA was determined *via* indirect methods (enzymatic hydrolysis). Yet higher glucuronidation rates were observed in other species (Meky et al., 2003; Warth et al., 2012). A comparably low activity of porcine UDP-glucuronosyltransferases, as shown by Maul et al. (2012), may account for this phenomenon.

In recent years, special focus has been put not only on the extent of glucuronidation, but also on species-dependent variations regarding the formation of different DON-GlcA isomers. Lattanzio et al. (2011) were the first to postulate the presence of two DON-GlcA conjugates in rat urine, while Warth et al. (2012) successfully identified DON-3-GlcA and DON-15-GlcA in urine of humans. In addition, evidence for the formation of further DON-GlcAs has been given (Maul et al., 2012; Nagl et al., 2012; Uhlig et al., 2013; Warth et al., 2013). To the best of our knowledge, the present study provides first *in vivo* data on the occurrence of different DON-GlcA isomers in pigs. Besides DON-3-GlcA, a second conjugate was detected in the urine of DON exposed piglets and was tentatively identified as DON-15-GlcA. While the latter was found to be the predominant conjugate in humans (Warth et al., 2012, 2013), ratios of DON-15-GlcA to DON-3-GlcA varied between individual piglets (0.5–2.2). Further studies are needed to investigate this aspect in more detail.

The high susceptibility of pigs to DON is often related to the species' poor ability to degrade DON to DOM-1 (Pestka 2007). In our experiment, DOM-1 could not be detected in urine or feces samples following DON indigestion. Moreover, none of the analytes included in our LC-MS/MS method seemed to be present in feces of piglets dosed with DON. While a complete lack of DOM-1 formation in swine is reported in literature (Sundstøl Eriksen et al., 2002), the absence of any toxins in feces may be due to several reasons. First, dilution of feces samples resulted in higher LODs and LOQs than in urine, thus raising the possibility that minimal toxin amounts were not detectable. More importantly, DON is primarily eliminated *via* urine in pigs. Since excreted toxin amounts in feces were reported to account for only 0.1–3% of the administered dose (Goyarts and Dänicke, 2006; Dänicke and Brezina, 2013), our results are not in conflict with previous studies.

#### 3.3.3. Oral D3G administration

In urine of piglets orally dosed with D3G, the masked mycotoxin itself, as well as DON, DON-3-GlcA, DON-15-GlcA and DOM-1 could

**Table 2**

Total amounts of recovered DON, D3G, DON-3-GlcA, DON-15-GlcA and DOM-1 in urine and feces of dosed piglets, respectively. Mean values  $\pm$  standard deviations (SD) are listed for the indicated time periods after oral administration of water, DON ( $0.25 \mu\text{mol/kg}$  b.w.) and D3G ( $0.25 \mu\text{mol/kg}$  b.w.) as well as after intravenous D3G application ( $0.03 \mu\text{mol/kg}$  b.w.). Since oral toxin administrations were incomplete in one of the animals, data from the respective piglet were excluded.

Treatment	Matrix	Time period (h)	DON $\pm$ SD (nmol)	D3G $\pm$ SD (nmol)	DON-3-GlcA $\pm$ SD (nmol)	DON-15-GlcA $\pm$ SD (nmol)	DOM-1 $\pm$ SD (nmol)
Water oral (n = 4)	Urine	0–8	$1.9 \pm 3.9$	n.d.	n.d.	n.d.	n.d.
		8–24	n.d.	n.d.	n.d.	n.d.	n.d.
	Feces	0–8	n.d.	n.d.	n.d.	n.d.	n.d.
		8–24	n.d.	n.d.	n.d.	n.d.	n.d.
DON oral (n = 3)	Urine	0–8	$880 \pm 220$	n.d.	$280 \pm 240$	$280 \pm 50$	n.d.
		8–24	$310 \pm 91$	n.d.	$170 \pm 65$	$62 \pm 40$	n.d.
	Feces	0–8	n.d.	n.d.	n.d.	n.d.	n.d.
		8–24	n.d.	n.d.	n.d.	n.d.	n.d.
D3G oral (n = 3)	Urine	0–8	$140 \pm 150$	$44 \pm 33$	$27 \pm 35$	$44 \pm 70$	$125 \pm 52$
		8–24	$330 \pm 130$	$15 \pm 4$	$45 \pm 11$	$100 \pm 34$	n.d.
	Feces	0–8	n.d.	n.d.	n.d.	n.d.	$44 \pm 41$
		8–24	n.d.	n.d.	n.d.	n.d.	n.d.
D3G i.v. (n = 4)	Urine	0–8	$12 \pm 24$	$410 \pm 27$	n.d.	n.d.	n.d.
		8–24	n.d.	$16 \pm 11$	n.d.	n.d.	n.d.
	Feces	0–8	n.d.	n.d.	n.d.	n.d.	n.d.
		8–24	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected (analyte concentration in all samples  $<$ LOD).

be found. Amounts of urinary excreted D3G were low, especially in the second time period ( $15 \pm 4$  nmol). A limited bioavailability of D3G has already been shown for rats (Nagl et al., 2012). In a human volunteer consuming a diet naturally contaminated with D3G ( $7 \mu\text{g}$  D3G/day), the masked mycotoxin could not be detected in urine at all (Warth et al., 2013). A likely explanation for these findings is provided by De Nijs et al. (2012) who showed that D3G is far less absorbed by human intestinal Caco-2 cells compared to DON. Taking into consideration both the limited bioavailability of D3G and its lower biological activity on the ribosomal level (Poppenberger et al., 2003) data indicate so far that D3G is less toxic than its parent toxin in mammals.

Yet major concerns have been raised regarding a possible hydrolysis of D3G with subsequent release of its aglycon in the digestive system. Results of several *in vitro* studies have supported these suggestions (Berthiller et al., 2011; Dall'Erta et al., 2013; Gratz et al., 2013), while *in vivo* reports on the issue are rare and only available for rats (Nagl et al., 2012; Veršilovskis et al., 2012). In the present experiment, DON as well as DON-15-GlcA and DON-3-GlcA were found in urine of piglets after oral D3G administration, confirming that D3G is cleaved to DON, absorbed and metabolized to DON-GlcA in pigs. Since these findings correlate well with those observed in rats (Nagl et al., 2012), hydrolysis of ingested D3G seems not to be species-dependent, but potentially a common feature of mammals in general. Notably, urinary excreted amounts of DON and DON-GlcA increased in samples of the second sampling period, with DON-1 solely being detectable 8–24 h after dosing (Table 1). Consistently, DON-1 was also found in feces samples of this time period. In pigs, this metabolite is known to be mainly formed in the hindgut (Dänicke et al., 2004a). With respect to the seemingly low absorption of D3G in the small intestine (De Nijs et al., 2012), it is likely that relevant amounts of the masked mycotoxin reach distal parts of the gut, where they are hydrolyzed to DON, further metabolized to DON-1 and absorbed. This scenario would explain the differences between the oral DON and D3G treatments regarding both the presence of DON-1 in investigated matrices as well as the delayed urinary excretion of D3G metabolites. However, further specific study designs are necessary to verify this hypothesis.

### 3.3.4. Intravenous D3G administration

The vast majority of intravenously administered D3G was excreted in unmetabolized form *via* urine in the first sampling period (Table 2). In contrast, only traces of urinary DON could be detected, whereas none of the other analytes was found. Berthiller et al. (2011) have shown that human cytosolic

$\beta$ -glucosidase, an enzyme expressed in liver, kidney, spleen and gut (Berrin et al., 2002), had no activity with D3G. In agreement, our data strongly indicate that hydrolysis of D3G is marginal after systemic absorption (or even not occurring at all if taking into account that low levels of urinary DON were also detected after water administration). As a consequence, we suppose that *in vivo* cleavage of D3G predominantly occurs in the digestive tract.

### 3.4. Total recoveries

The quantitative urinary recovery of DON allows an appropriate estimation of the toxin's bioavailability in pigs (Goyarts and Dänicke, 2006). After oral administration of DON,  $84.8 \pm 9.7\%$  of the administered dose were detected in urine within 24 h in total (Fig. 3). Unmetabolized DON accounted for  $51.4 \pm 6.3\%$ , while DON-3-GlcA and DON-15-GlcA reflected  $19.0 \pm 6.8\%$  and  $14.5 \pm 1.8\%$  of the applied toxin dose, respectively. The high oral bioavailability of DON is in line with literature reports, where urinary DON recoveries between 44 and 82% were assessed after single oral/intragastric DON administration (Dänicke et al., 2004b; Prelusky et al., 1988). Furthermore, urinary recoveries determined in our study are comparable to those observed in humans (summarized by Turner et al., 2012) underlining once more the similarities between those species in regard to DON metabolism.

Following oral D3G application, the overall amount of recovered analytes reflected  $42.1 \pm 8.5\%$  of the administered toxin dose. Thereof, urinary excreted metabolites represented  $40.3 \pm 10.0\%$ , whereas elimination *via* feces accounted for only  $1.8 \pm 1.6\%$ . Although the primary excretion route of D3G seems to differ between pigs and rats, the low oral bioavailability of the masked mycotoxin applies for both species: urinary D3G accounted for  $2.6 \pm 1.4\%$  in our study, while amounts of  $0.3 \pm 0.1\%$  were reported for rats (Nagl et al., 2012). DON, DON-3-GlcA, DON-15-GlcA and DON-1 in piglets' urine reflected  $21.6 \pm 3.7\%$ ,  $3.4 \pm 2.0\%$ ,  $6.8 \pm 4.1\%$  and  $5.9 \pm 2.9\%$  of the administered dose, respectively. Despite the nearly complete hydrolysis and metabolization of D3G, the absorption of the formed metabolites seems to be limited. In comparison to DON treatment, the proportion of urinary excreted metabolites was reduced by a factor of 2, approximately. DON is predominantly absorbed in proximal parts of the small intestine in pigs (Dänicke et al., 2004a), whereas *in vitro* experiments indicate that D3G is hydrolyzed thereafter (Berthiller et al., 2011; Dall'Erta et al., 2013). Thus, the obtained *in vivo* data are not entirely surprising. Still, also non-absorbed hydrolysis products of D3G could exhibit adverse health

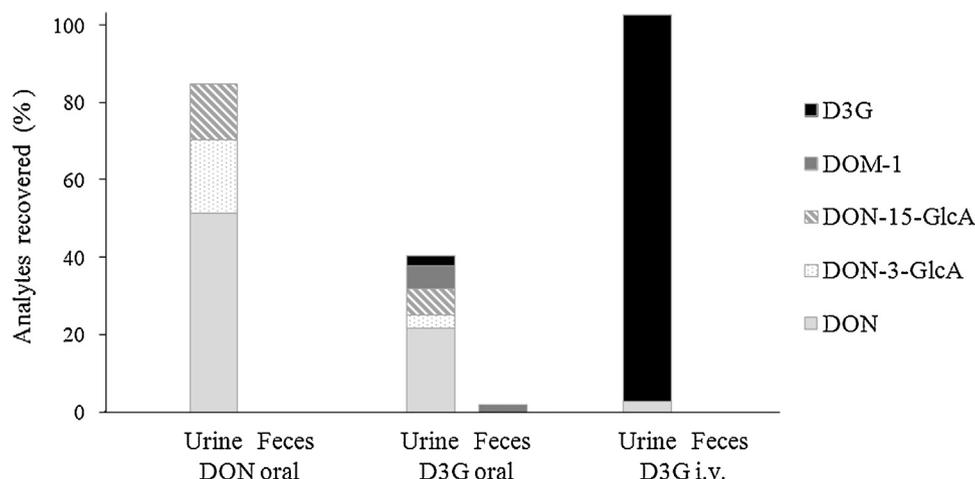


Fig. 3. Excretion of DON, D3G and their metabolites in urine and feces of toxin exposed piglets. Values are expressed as DON equivalent percentages of the administered dose.

effects. For example, DON was shown to induce intestinal damage by alteration of tight junction protein expression and decrease of the barrier function (Pinton et al., 2009). Data dealing with possible effects of D3G and its degradation products on the gut health are lacking so far.

Yet another question of interest is the discrepancy in the overall recovered toxin amounts between oral DON and D3G treatment. One explanation might be an incomplete elimination of D3G by 24 h, as indicated by the considerable amounts of urinary excreted analytes 8–24 h after dosing. Thus, the short sampling period represents a limitation of our study. Moreover, we cannot ensure that the employed biomarker method includes all metabolites and degradation products which are actually formed of D3G *in vivo*. For example, novel sulfated DON metabolites have been identified in rats and chicken just recently (Wan et al., 2014). Furthermore, some intestinal bacteria may be able to use the phosphorelay uptake system and convert D3G into DON-3-phosphoglucoside, but are inefficient to cleave this metabolite into DON and glucose-phosphate. Currently, no standard is available for DON-3-phosphoglucoside. Also the formation of other (fecal) metabolites cannot be excluded. However, to encounter this problem to some extent, we investigated the presence of DOM-1-glucuronide (DOM-1-GlcA) in urine samples. Enzymatic treatment resulted in an increase of DOM-1 concentrations in urine of piglets orally dosed with D3G by a factor of 3.5 on average, thus indicating the presence of DOM-1-glucuronide. Due to a lack of a suitable standard, this metabolite was not quantified in our study. In comparison, an extensive metabolism of the masked mycotoxin after its systemic absorption seems rather unlikely, since almost the complete amount of intravenously administered D3G was recovered in unmetabolized form in urine ( $99.9 \pm 8.9\%$  out of  $102.4 \pm 5.4\%$  total recovery). In the future, the investigation and identification of unaccounted D3G metabolites, especially of those formed by gut microbiota, using high resolution mass spectrometers will be a major task.

In conclusion, we demonstrated that ingested D3G is nearly completely hydrolyzed in pigs, but only partially absorbed. Results indicate that the cleavage predominantly occurs in the digestive tract, while the toxin seems to be rather stable after systemic absorption. Compared to DON, the proportion of urinary excreted metabolites after oral D3G administration was reduced by a factor of 2, approximately. Based on these data we presume that D3G is less bioavailable than its parent toxin in pigs and therefore of lower toxicological relevance. However, the bioavailability of D3G in pigs may increase after chronic exposure *via* feed, as it has already been shown for DON (Goyarts and Dänicke, 2006). Clarification of this aspect will be also important regarding the risk assessment of D3G and its potential implementation in regulatory limits. Furthermore, it should be emphasized that D3G may exhibit biological activity on its own. Therefore, future studies should for instance address possible emetic effects of the masked mycotoxin or its influence on intestinal gut health.

#### Conflict of interest

The authors declare that there are no conflicts of interest.

#### Transparency document

The Transparency document associated with this article can be found in the online version.

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