Porcine small and large intestinal microbiota rapidly hydrolyze the masked mycotoxin deoxynivalenol-3-glucoside and release deoxynivalenol in spiked batch cultures *in vitro*.

Running title: Porcine intestinal microbiota hydrolyze DON3Glc

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Keywords: deoxynivalenol-3-glucoside, pig, microbiota, masked mycotoxin, release, toxicity, trichothecene

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Abstract

Mycotoxin contamination of cereal grains causes well-recognized toxicities in animals and humans, but the fate of plant-bound masked mycotoxins in the gut is less well understood. Masked mycotoxins have been found to be stable under conditions prevailing in the small intestine, but are rapidly hydrolyzed by fecal microbiota. This study aims to assess the hydrolysis of the masked mycotoxin deoxynivalenol-3-glucoside (DON3Glc) by microbiota of different regions of the porcine intestinal tract.

Intestinal digesta samples were collected from the jejunum, ileum, caecum, colon and feces of 5 pigs and immediately frozen under anaerobic conditions. Sample slurries were prepared in M2 culture medium, spiked with DON3Glc or free DON (2 nmoles/mL) and incubated anaerobically for up to 72 hours. Mycotoxin concentrations were determined using LC-MS/MS and microbiota composition was determined using qPCR methodology.

Jejunal microbiota hydrolyzed DON3Glc very slowly, while samples from the ileum, caecum, colon and feces rapidly and efficiently hydrolyzed DON3Glc. No further metabolism of DON was observed in any sample. Microbial load and microbiota composition was significantly different in the ileum, but similar in caecum, colon and feces.

Importance

Results from this study clearly demonstrate that the masked mycotoxin DON3Glc is hydrolyzed efficiently in the distal small intestine and large intestine of pigs. Once DON is released, toxicity and absorption in the distal intestinal tract are likely to occur *in vivo*. This study further supports the need to include masked metabolites into mycotoxin risk assessments and regulatory actions for feed and food.

Introduction

Mycotoxin contamination of agricultural commodities is an intractable problem globally. In temperate climates *Fusarium* fungi comprise the most important mycotoxin producers and are particularly prevalent in small grain cereals such as wheat and barley as well as maize. The major groups of *Fusarium* mycotoxins include trichothecenes, zearalenone and fumonisins [1]. In addition to the well described trichothecenes deoxynivalenol (DON), nivalenol, T2 toxin and HT2 toxin, cereals have been found to be co-contaminated with plant-derived mycotoxin metabolites, so-called masked mycotoxins. In response to fungal infection and mycotoxin production, the plant's own phase II metabolic enzymes conjugate mycotoxins with small molecules such as glucose, glutathione or sulphate and sequester these masked mycotoxins into the plant cell vacuole (for review see [2-4]). Mycotoxins and masked mycotoxins are stable compounds withstanding processing into various cereal products and are carried over into finished food and feed. Once ingested, mycotoxins have been shown to be rapidly absorbed in the small intestine of humans and various animal species and exert their toxicities either locally on the gut epithelium (e.g. trichothecenes) or systemically (e. g. zearalenone) [1,4-6]. Masked mycotoxins such as DON-3-β,D-glucoside (DON3Glc) on the other hand, are far less toxic compared to their free parent mycotoxins and are not absorbed intact [7-9]. Hence masked mycotoxins are transported into the distal parts of the intestine intact where the intestinal microbiota (as studied using fecal samples) rapidly hydrolyze masked mycotoxins and release free mycotoxins [7,10-12]. Microbial metabolism experiments have also demonstrated further metabolism of DON to de-epoxy DON (DOM-1) by microbiota samples derived from chickens, pigs, and some humans [10,13,14]. This purely microbial metabolite, DOM-1, is not toxic [15] and can be found in urine of some humans [10,16,17] and pigs [18] hence confirming its production and colonic absorption i*n vivo*.

In pigs oral bioavailability and absorption of DON3Glc is significantly lower and slower, compared to DON [18,19]. The delay in DON3Glc absorption and the fact that only free DON and no DON3Glc are found in plasma and urine, confirm that the hydrolysis and absorption occur in the more distal parts of the intestinal tract compared to free DON. Microbial de-epoxidation of DON or DON3Glc by pig microbiota has been found in some studies [14,18] but not in others [19].

All studies published to date have used fecal samples from pigs or humans to determine microbial hydrolysis and metabolism of mycotoxins. However, microbial metabolism of mycotoxins would need to occur in more proximal parts of the intestinal tract to release mycotoxin metabolites and allow intestinal absorption and/or potential colonic toxicity to occur. Therefore, the aim of this study was to investigate the capacity of intestinal microbiota derived from different regions of the small and large intestine of pigs to degrade masked mycotoxins. For this study, DON3Glc was used as model mycotoxin as it is commercially available.

Materials and Methods

The following mycotoxin standards were used in this study: DON as powder (Molekula, Gillingham, UK); DON, and DON3Glc in acetonitrile (Romer Labs, Runcorn, UK) and DOM-1 in acetonitrile (Sigma-Aldrich Ltd, Poole, UK).

Animals and ethical approval

Five crossbred castrated male pigs, weaned at four weeks were bred in the animal facility of the INRA ToxAlim Laboratory (Toulouse, France). The experiment was conducted under the

authorization of the French ministry of Higher Education and Research after approval by the Ethics committee of Pharmacology-Toxicology of Toulouse-Midi-Pyrénées (Toxcométhique, N°: TOXCOM/0163 PP), in accordance with the European Directive (2010/63/EU) on the protection of animals used for scientific purposes. Feed and water were provided ad libitum throughout the experimental period. Pigs were fed for 4 days with starter diet and then with a commercial diet "STIMIO" for growing pigs (Evialis, Longue Jumelles, France), the feed composition is summarized in Table 1. As the presence of antibiotics or probiotics in feed can alter the composition of the luminal and mucosa-associated microbiota [20] non-supplemented feed was used. Pigs were maintained until 57 days of age as the pig intestinal flora is stable between at least 48 and 70 days of age [21]. Then, they were subjected to electronarcosis and euthanized by exsanguination [22]. The intestinal tract was removed from each carcass and sections of the jejunum, ileum, caecum and colon were dissected. Five millilitres of intestinal digesta content from each gut section was collected separately into sterile Wheaton bottles. Feces (5 mL) was sampled directly from the pen. Ten mL of a sterile mixture of 70% phosphate buffered saline (pH 7.4) /30% glycerol bubbled with CO_2 were added into each vial. Vials were sealed and the headspace flushed with CO_2 before being stored at -20°C.

Microbial batch culture experiments

After defrosting and vortexing, 15 mL of slurry were centrifuged at 2000 × g for 5 minutes. Supernatant was discarded and the remaining pellet was purged with CO_2. At this stage, two 1 mL aliquots were removed from each sample and stored in sample Matrix tubes at -70°C for subsequent DNA extraction. The remaining slurry was diluted 1/10 with anaerobic M2 medium as described before [10], placed in a shaking water bath (37°C, 100 rpm) in a sealed Wheaton bottle for 1 hour and 1 mL aliquots were moved to sterile screw-capped Hungate tubes. Slurry aliquots were spiked with individual mycotoxins (2 nmol/mL of DON, DON3Glc or DOM-1)

and incubated anaerobically at 37°C for intervals between 0-72 hours. This wide range of incubation times was chosen to reflect both the short transit time in the small intestine (early time points) and the long transit time in the large intestine (late time points). Following incubation, 3 mL acetonitrile were added to each sample and samples were centrifuged for 5 minutes at 2000 × *g*. Supernatants were evaporated under N_2 at 50°C, reconstituted with 1 mL of water and passed through C18 solid phase extraction columns (Agilent, Wokingham, UK). Samples were eluted with 3 mL methanol, evaporated under N_2 at 50°C, reconstituted into 1 mL of 50% aqueous methanol, and analysed for DON, DON3Glc and DOM-1 using LC-MS/MS. Blank digesta incubations (omitting spiking with mycotoxins) were included in each experiment to ensure that all digesta samples were free of mycotoxin residues. Furthermore, DON3Glc or DON (2 nmoles/mL) were spiked individually into bacteria-free M2 culture media (in duplicates) and incubated for 72 hours to ensure stability of DON3Glc and DON under incubation conditions in the absence of bacteria. Both compounds were stable with recoveries of 100.7± 4.7% and 102.8± 1.9%, respectively after 72 hours. To further assess DON stability during incubation, digesta samples were spiked with DON (2 nmoles/mL) and incubated for 0 to 72 hours. This experiment showed no mass loss of DON (recovery up to 119% of dose added), suggesting no binding of DON or further metabolism by microbes or any other digesta constituents. Each experiment also included digesta controls (in duplicate) spiked with DON3Glc, DON or DOM-1 individually, which were not incubated and immediately processed further (i.e. time 0) to account for potential matrix effects in mycotoxin detection. Mycotoxins detected in time 0 samples were set as 100% and all other results were calculated as % of time 0.

QPCR analysis of microbial composition

Microbiota composition was analyzed using DNA extracted from untreated digesta samples (without mycotoxin spiking) derived from ileum, caecum, colon and feces of experimental pigs. Jejunal samples did not yield sufficient DNA to perform qPCR analysis. DNA was extracted from 1 mL of digesta slurry using the FastDNA™ Kit for Soil, (MP Biomedicals, Santa Ana, CA, USA) following the manufacturers' instructions, and quantified using Qubit®dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). QPCR was performed using primers described in Table 2. The quantification of total bacteria, *Prevotella spp.*, *Bacteroides spp.*, Ruminococcaceae, Lachnospiraceae and Negativicutes, *Lactobacillus spp.*, enterobacteria and bifidobacteria was performed as described before [24] using a Bio-Rad CFX384 Real Time system and Bio-Rad CFX Manager Software 3.0 (Bio-Rad Laboratories, Watford, UK). DNA concentrations were standardised to 1 ng per well and standard curves consisted of dilution series of amplified bacterial 16S rRNA genes from reference strains as described previously [30]. Samples and standards were run in duplicate and 5 ng/µL Herring Sperm DNA (Promega, Southampton, UK) was included in all reactions for stabilization. The efficiencies of standard curves ranged from 92.6 – 104.7% and R^2 values ranged from 0.993 – 0.999 across all primers used.

LC-MS/MS analysis

The liquid chromatography analysis of the mycotoxins was performed on an Agilent 1200 HPLC system (Agilent Technologies, Wokingham, UK) fitted with an Agilent Zorbax 5 µm, 150 mm × 4.6 mm C18 column. The method parameters were described previously [10]. Mycotoxins were detected on a Q-Trap 4000 triple quadrupole mass spectrometer (AB Sciex, Warrington, UK) fitted with a Turbo Ion Spray™ (TIS) source. The transitions for DON, DOM-1 and DON3Glc from microbial incubations were: 355.1 → 265.1, 339.1 → 249.1 and

517.3 → 427.3, respectively. Calibration curves for each metabolite ranged from 0.25 to 2 nmol/mL.

Statistical analysis

The time course over 72 hours of the DON and DON3Glc hydrolysis results from Figure 1 (expressed as % of dose) were used to calculate the area under the curve (AUC, % × hr) for each animal and each intestinal section individually. Bacterial count data were log-transformed to meet requirements of constant variance and normality (based on visual inspection of residual plots). These data were then analysed by ANOVA, with animal as random effect and tissue as fixed effect. When the effect of tissue was significant ($P<0.05$), tissues were compared by post hoc t-test based on the ANOVA output. The colon sample of one animal was excluded from the statistical analyses due to failure of the qPCR assay. All analyses were carried out using Genstat 17 Release 17.1 (Lawes Agricultural Trust, VSN international Ltd, Hemel Hempstead, UK). A P-value < 0.05 was regarded significant. Results are presented as mean±SEM, based on spread between animals.

Results

This study was conducted to assess the metabolism of DON and DON3Glc by porcine microbiota derived from different regions of the intestinal tract. Results show that detoxification of DON to DOM-1 did not occur in any animal or any gut region (Table 3). No trace of DOM-1 was detectable in any of the samples (data not shown), and recovery of DON ranged from 87-119% of dose following incubation over 24-72 hours.

Microbial hydrolysis of DON3Glc was efficient in all pigs and occurred at all intestinal regions tested (Figure 1). In the jejunum, DON3Glc hydrolysis was slowest and free DON was

first observed after 24 hours of incubation, increasing to a maximum of 1–41% of the added DON3Glc dose after 72 hours. The ileal microbiota was more efficient in DON3Glc hydrolysis releasing 60±18% of the dose as free DON after 24 hours of incubation. Microbiota of the large intestine hydrolyzed DON3Glc more rapidly with 2 and 3% of the dose detectable as free DON in caecum and colon incubations after 2 hours increasing to 8 and 14% after 6 hours of incubation. Fecal microbiota were most efficient in hydrolyzing DON3Glc with only 4±6% of the dose left as DON3Glc after 9 hours of incubation.

The results from the DON3Glc hydrolysis time course experiments (Figure 1) were used to calculate the area under the curve (AUC) for each individual animal and each intestinal region for DON3Glc (Figure 2, top panel) and DON (bottom panel). DON3Glc hydrolysis rates were slowest for all animals in jejunal samples, as indicated by the highest AUC for DON3Glc curves and the lowest AUC for DON curves. Ileal DON3G hydrolysis was significantly faster in all animals ($P<0.05$) than jejunal hydrolysis, but slower ($P<0.05$) than rates observed in the large intestine. No differences were observed between DON3Glc hydrolysis rates in caecum, colon and fecal samples.

Microbiota composition was analyzed using DNA extracted from untreated digesta samples (without mycotoxin spiking) derived from ileum, caecum, colon and feces of experimental pigs. Total bacterial load showed a tendency ($P=0.057$) towards differences between intestinal regions, with a lower log count in the ileum compared to the caecum and colon ($P<0.05$) (Figure 3). Log counts of *Bacteroides* spp., *Prevotella* spp., Ruminococcaceae, Lachnospiraceae and Negativicutes were all lower in the ileum ($P<0.05$) but did not differ between caecum, colon and feces. In the ileum members of the phylum Firmicutes dominated the microbiota with lactobacilli forming the largest portion of bacteria. However, most bacteria in the ileum were not identified with the primers used, suggesting that the ileum harbors bacteria out with the groups covered here.

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212 Discussion

213 The current study was conducted to assess the microbial metabolism of the masked mycotoxin
214 DON3Glc and the free form DON by intestinal microbiota derived from different regions of
215 the small and large intestine of pigs. We found no evidence of microbial de-epoxydation of
216 DON to DOM-1 in any digesta sample. Similarly, Eriksen and colleagues found no DOM-1
217 production in ileal or fecal samples from 5 experimental pigs even though DOM-1 production
218 was reported in pigs from commercial farms [14]. Interestingly, 4 of these 5 animals acquired
219 the microbiota capable of DOM-1 production after they were exposed to feces of DOM-1
220 producing animals. This suggests that the microbes capable of DON de-epoxydation are
221 acquired from the environment and confirms that ingestion of DON-contaminated feed may
222 alter the intestinal microbiota [31,32].

223 The study presented here demonstrates that microbiota derived from the porcine small
224 intestine efficiently hydrolyze the masked mycotoxin DON3Glc and release free DON *in vitro*.
225 Furthermore, microbiota from the porcine caecum, colon and feces hydrolyze DON3Glc
226 equally efficiently. Upon ingestion, DON3Glc has been found to be not toxic (in pig intestinal
227 explants [8]) and is not absorbed intact in pigs, but free DON and further metabolites are
228 detectable in plasma and urine. DON3Glc absorption in pigs (as DON) is less efficient
229 compared to free DON (16% vs 81% of dose absorbed after 8 hours [19]) and also slower than
230 DON (42 vs 84% of dose excreted in urine after 24hours [18]). These findings suggest
231 continuous, slow release of DON from DON3Glc prior to absorption, which would be in line
232 with microbial hydrolysis beginning after 6 or 9 hours incubation as reported here.

This slow and continuous DON release from DON3Glc exposure may result in toxicities in the more distal regions of the intestine compared to DON dosed animals. There is some evidence that binding of DON to a clay-based feed additive results in DON exerting its intestinal toxicity (disruption of intestinal barrier function, induction of oxidative stress) in the more distal part of the small intestine in chickens when compared to free DON, although colonic tissue was not evaluated in this study [33]. This suggests that binding of DON can lead to the intestinal toxicity being shifted to more distal intestinal regions and it can be hypothesized that plant-bound DON3Glc could act as delivery mechanism to the ileum and colon where microbial hydrolysis will lead to DON exposure and potential toxicity. Upon ingestion of DON3Glc, it would be interesting to determine the absorption and the effect of DON in the large intestine.

Microbiota profiling demonstrated that microbiota from the caecum, colon and feces were dominated by *Prevotella* spp., followed by Ruminococcaceae, Lachnospiraceae and Negativicutes. This is in agreement with literature suggesting Bacteroidetes and Fimicutes to be the dominant phyla in the large intestine and feces of pigs [34-36]. Enterobacteria represented a substantial group in the small and large intestine of only one pig, whereas *Bacteroides* spp. and bifidobacteria did not represent major groups in any animal or gut site. This is in contrast with published work [37] reporting *Bacteroides* spp. to be a major group in porcine feces. The current study focused on quantitative and qualitative analysis of the intestinal microbiota of the porcine intestine, but did not identify specific bacterial groups involved in the hydrolysis of DON3Glc. Published work has identified bacteria from very different genera and phyla (lactobacilli, enterococci, bifidobacteria) that are capable of hydrolyzing DON3Glc and other masked mycotoxins [38,39] and future studies are required to understand their contribution to hydrolysis is mixed microbial communities and *in vivo*.

257 The human intestinal microbiota possess several glycosyl hydrolase genes [40] and
258 human fecal microbiota are known to hydrolyse DON3Glc [7,10,11]. It is therefore likely that
259 DON3Glc hydrolysis occurs in the human inestine, but future experiments are required to
260 provide evidence. The fact that the microbial metabolite DOM-1 is present in human urine
261 [10,16,17] further supports the hypothesis that microbial mycotoxin metabolism and absorption
262 occur *in vivo* in humans.

263 In conclusion, the present study demonstrates that masked mycotoxins can contribute
264 to mycotoxin exposure following rapid, efficient and non-specific hydrolysis by intestinal
265 microbiota of the distal small intestine and the large intestine. Potential specific toxicities of
266 microbial mycotoxin release in the distal intestine remain to be investigated in future studies.

267

268 Acknowledgement

269 This study was supported by the Scottish Government Rural and Environment Science and
270 Analytical Services division (RESAS) and by the French Agence Nationale de la Recherche
271 (project ANR-13-CESA-0003-03). We thank Anne-Marie Cossalter for her excellent technical
272 assistance with pigs.

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406 Figure legends

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408 Figure 1. Hydrolysis of DON3Glc and release of free DON by porcine intestinal microbiota
409 form different regions of the small and large intestine over 0–72 hours. Results are presented
410 as average of 5 animals±SEM.

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412 Figure 2. Individual differences in DON3Glc hydrolysis (top panel) and DON release (bottom
413 panel) by intestinal microbiota of 5 animals. Data from time course experiments were
414 summarized by area under the curve (AUC) for each individual animal and gut site. Effect of
415 tissue was significant ($P<0.001$, ANOVA) for both DON3Glc and DON. Tissues that do not
416 share a superscript are significantly different ($P<0.05$, post-hoc t-test).

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418 Figure 3. Microbial community analysis in porcine digesta samples from different regions of
419 the small and large intestine. Results are presented as averages of 5 animals±SEM for ileum,
420 caecum and feces and average of 4 animals±SEM for colon samples. Within those bacterial
421 groups for which the effect of tissue was significant ($P<0.05$, ANOVA), tissues that do not
422 share a superscript are significantly different ($P<0.05$).

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424 Table 1. Summary of feed composition.

Food constituent	**Unit**	**Oligo elements**	**mg/kg**
Raw proteins	17 %	Iron	86
Raw fat	2.5 %	Copper	160
Raw ashes	4.5 %	Manganese	40
Crude fiber	4.5 %	Zinc	110
Phosphorus	0.55 %	Iodine	1
Calcium	0.65 %	Selenium	0.3
Sodium	0.2 %		
Lysine	11.9 g/kg		
Methionine	3.6 g/kg		
Additives	**Units/kg**	**Enzymes**	**Units/kg**
E672 A vitamin	12000	Endo 1, 3 (4) beta glucanase	125 U
E671 D3 vitamin	2000	Endo 1, 4 beta xylanase	87 U
3a700 E vitamin E	60	Phytase	1880 U

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429 Table 2. Summary of group-specific qPCR primers.

Target group	Sequence	T	Amplicon	Reference strain	Ref.
Universal	GTGSTGCAYGGYYGTCGTCA	60	141	*Ruminococcus bromii* L2-63	[23]
	ACGTCRTCCMCNCCTTCCTC				
Prevotella spp.	CRCRCRGTAAACGATGGATG	65	105	*Prevotella copri* DSM18205	[24]
	TTGAGTTTCACCGTTGCCGG				
Bacteroides spp.	GCTCAACCKTAAAATTGCAGTTG	63	110	*Bacteroides thetaiotamicron* B5482	[24]
	GCAATCGGRGTTCTTCGTG				
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA	60	341	*Lactobacillus reuteri* DSM20016	[25]
	CACCGCTACACATGGAG				[26]
Bifidobacteria	TCGCGTCYGGTGTGAAAG	60	128	*Bifidobacterium adolescentis* DSM20083	[23]
	GGTGTTCTTCCCGATATCTACA				
Enterobacteria	GACCTCGCGAGAGCA	60	180	*Escherichia coli* XL1Blue	[27]
	CCTACTTCTTTTGCAACCCA				
Cluster IV Ruminococcaceae family	GCACAAGCAGTGGAGT[1]	60	241	*R. bromii* L2-63	[28]
	GCACAAGCGGTGGATT[1]				
	CTTCCTCCGTTTTGTCAA				
Cluster IX Negativicutes class	GTTGTCCGGAATYATTGGGC	63	321	*Megasphaera elsdenii* LC1	[29]
	ATTGCGTTAACTCCGGCACA[2]				
	ATTGCGTTAACTCCGGCACG[2]				
Cluster XIVa Lachnospiraceae family	CGGTACCTGACTAAGAAGC	60	429	*Roseburia hominis* A2-183	[30]
	AGTTTYATTCTTGCGAACG				

430 Both primers ([1]forward primers for cluster IV, [2]reverse primers for cluster IX primers) were
431 used together at equimolar concentration. T Annealing temperature.
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437 Table 3. Recovery (% of dose 2 nmol/mL) of DON from microbial incubations.

Time	Jejunum	Ileum	Caecum	Colon	Feces
0 hr	100.3 (±0.3)	102.7 (±1.2)	98.6 (±1.1)	100.5 (±0.3)	99.9 (±0.1)
24 hr	103.9 (±6.7)	97.2 (±8.4)	94.0 (±9.3)	99.6 (±8.3)	119.4 (±5.7)
48 hr	87.3 (±4.3)	90.0 (±6.1)	113.9 (±6.2)	103.9 (±9.3)	114.6 (±8.7)
72 hr	90.3 (±6.2)	91.8 (±6.6)	116.0 (±6.5)	108.1 (±9.0)	113.6 (±7.8)

438 Results presented as average of 5 animals±SEM.