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

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Running title: Porcine intestinal microbiota hydrolyzes 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 mycotoxin 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 intestine.

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 (2nmoles/mL) and incubated anaerobically 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 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 enzyme systems 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, confirms the hydrolysis and absorption to 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 human 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 during four 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 out 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 1mL 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 was 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 and 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. Digesta samples spiked with DON (2nmoles/mL) were incubated between 0 and 72 hours and 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. 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 (Life Technologies). 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, City, Country). 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]. Sample and standards were run in duplicate and 5ng/µL Herring Sperm DNA (Promega) 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 72h 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 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 was 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

188 intestine hydrolyzed DON3Glc more rapidly with 2 and 3% of the dose detectable as free DON
189 in caecum and colon incubations after 2 hours increasing to 8 and 14% after 6 hours of
190 incubation. Fecal microbiota were most efficient in hydrolyzing DON3Glc with only 4±6% of
191 the dose left as DON3Glc after 9 hours of incubation.

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

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

211

Discussion

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

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

This slow and continuous DON release may result in toxicities in the more distal regions of the intestine than observed in 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 [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 hydrolysis. 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*.

The human intestinal microbiota possess several glycosyl hydrolase genes [41] and human fecal microbiota are known to hydrolyse DON3Glc [7,10,11]. It is therefore likely that DON3Glc hydrolysis occurs in the human inestine, but future experiments are required to provide evidence. The fact that the microbial metabolite DOM-1 is present in human urine

260 [10,16,17] further supports the hypothesis that microbial mycotoxin metabolism and absorption
261 occur *in vivo* in humans.

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

266

267 Acknowledgement

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

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409 Figure legends

410

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

414

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

420

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

426

427 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)	**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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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				

Both primers ([1]forward primers for cluster IV, [2]reverse primers for cluster IX primers) were used together at equimolar concentration. T Annealing temperature.

440 Table 3. Recovery (% of dose 2 nmol/mL) of DON from microbial incubations. Results
441 presented at average of 5 animals±SEM.

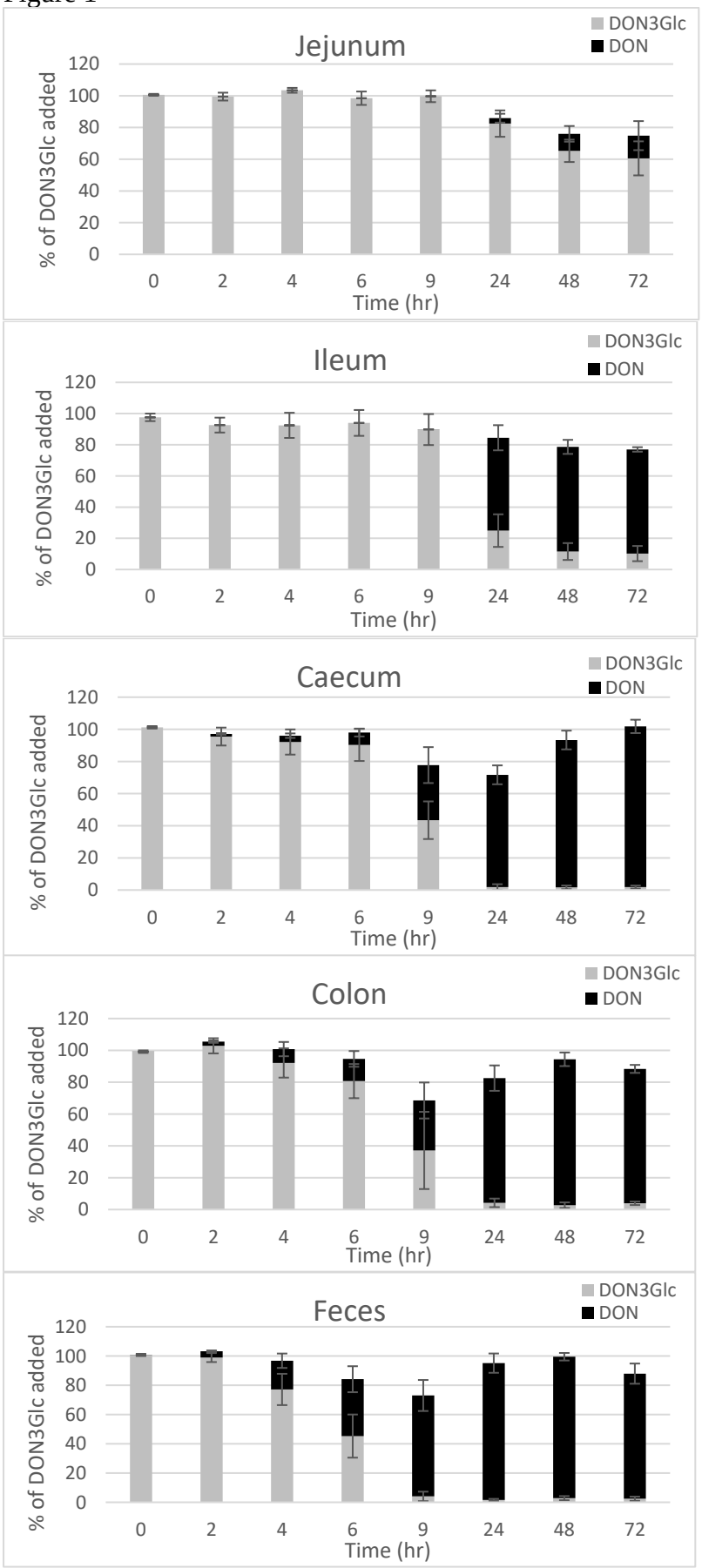
442

Time	Jejunum	Ileum	Caecum	Colon	Faeces
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)

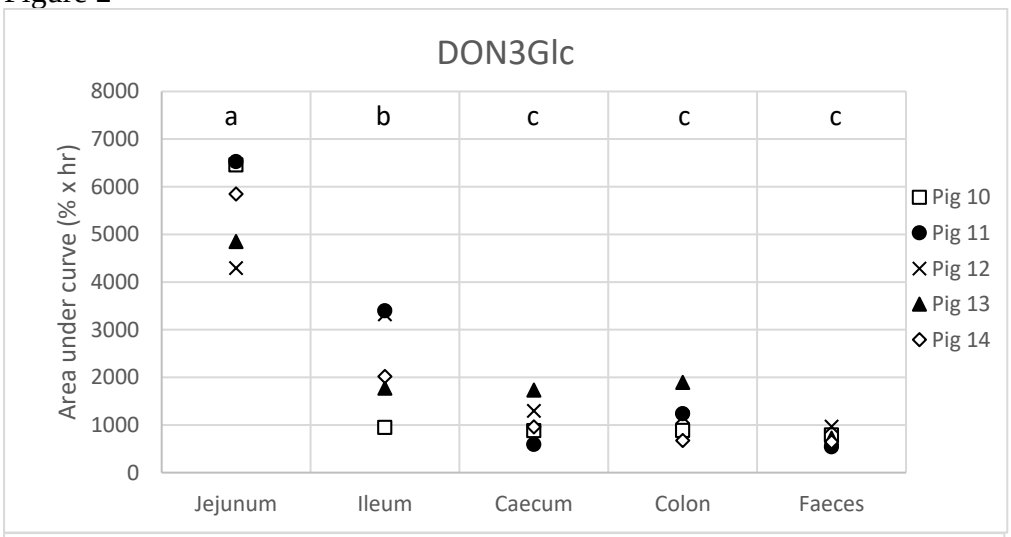
443

444

Figure 1


Jejunum
DON3Glc
DON
% of DON3Glc added
120
100
80
60
40
20
0
0 2 4 6 9 24 48 72
Time (hr)
Ileum
DON3Glc
DON
% of DON3Glc added
120
100
80
60
40
20
0
0 2 4 6 9 24 48 72
Time (hr)
Caecum
DON3Glc
DON
% of DON3Glc added
120
100
80
60
40
20
0
0 2 4 6 9 24 48 72
Time (hr)
Colon
DON3Glc
DON
% of DON3Glc added
120
100
80
60
40
20
0
0 2 4 6 9 24 48 72
Time (hr)
Feces
DON3Glc
DON
% of DON3Glc added
120
100
80
60
40
20
0
0 2 4 6 9 24 48 72
Time (hr)

451 Figure 2



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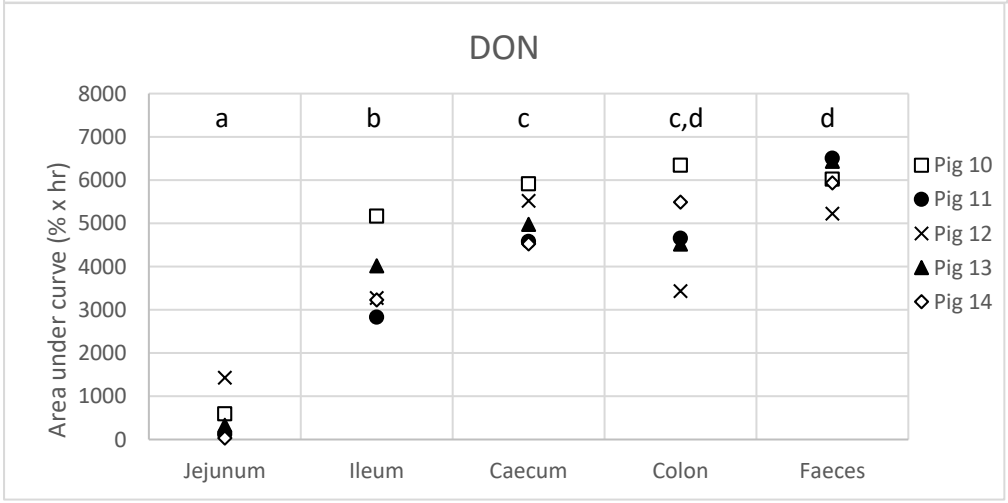

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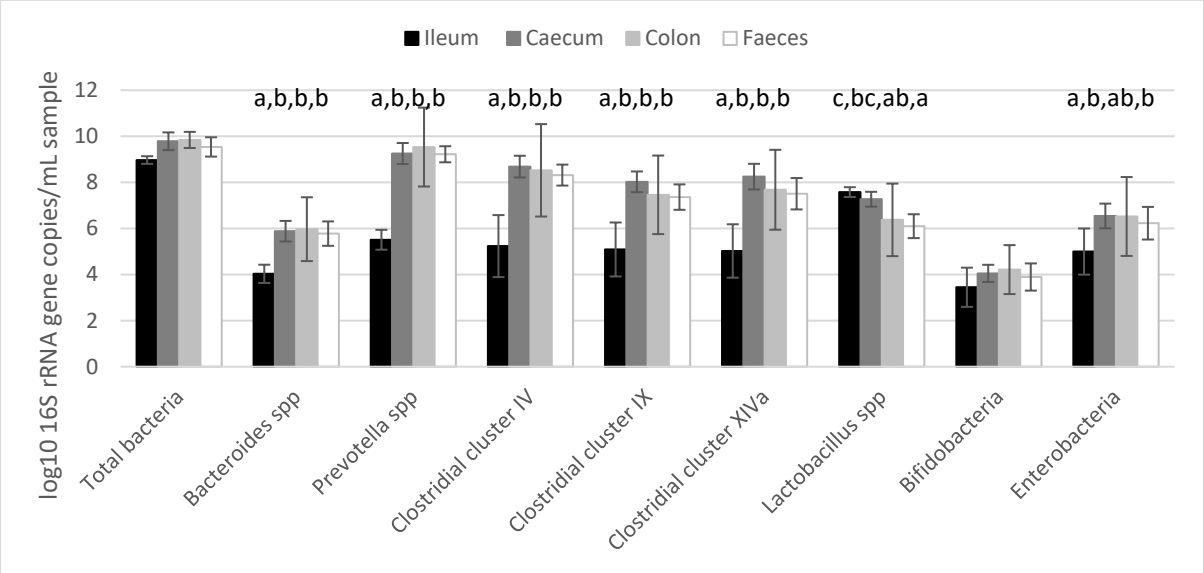
458 Figure 3



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