The Mycotoxin Deoxynivalenol Predisposes for the Development of Clostridium perfringens-Induced Necrotic Enteritis in Broiler Chickens

Gunther Antonissen1,2*, Filip Van Immerseel1,9, Frank Pasmans1,9, Richard Ducatelle1, Freddy Haesebrouck1, Leen Timbermont1, Marc Verlinden1, Geert Paul Jules Janssens3, Venessa Eeckhaut1, Mia Eeckhout5, Sarah De Saeger5, Sabine Hessenberger6, An Martel11†, Siska Croubels2†

1 Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, 2 Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, 3 Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, 4 Department of Applied Biosciences, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium, 5 Department of Bio-analysis, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium, 6 Biomin Research Center, Tulln, Austria

Abstract

Both mycotoxin contamination of feed and Clostridium perfringens-induced necrotic enteritis have an increasing global economic impact on poultry production. Especially the Fusarium mycotoxin deoxynivalenol (DON) is a common feed contaminant. This study aimed at examining the predisposing effect of DON on the development of necrotic enteritis in broiler chickens. An experimental Clostridium perfringens infection study revealed that DON, at a contamination level of 3,000 to 4,000 μg/kg feed, increased the percentage of birds with subclinical necrotic enteritis from 20% to 26.6% to 47% (P<0.001). DON significantly reduced the transepithelial electrical resistance in duodenal segments (P<0.001) and decreased duodenal villus height (P<0.014) indicating intestinal barrier disruption and intestinal epithelial damage, respectively. This may lead to an increased permeability of the intestinal epithelium and decreased absorption of dietary proteins. Protein analysis of duodenal content indeed showed that DON contamination resulted in a significant increase in total protein concentration (P=0.023). Furthermore, DON had no effect on in vitro growth, alpha toxin production and netB toxin transcription of Clostridium perfringens. In conclusion, feed contamination with DON at concentrations below the European maximum guidance level of 5,000 μg/kg feed, is a predisposing factor for the development of necrotic enteritis in broilers. These results are associated with a negative effect of DON on the intestinal barrier function and increased intestinal protein availability, which may stimulate growth and toxin production of Clostridium perfringens.

Introduction

Worldwide, necrotic enteritis (NE) leads to important production losses, increased feed consumption and mortality rates, and a reduced welfare of broiler chickens [1-4]. The causative agent of NE is Clostridium perfringens, a Gram-positive spore forming bacterium which occurs ubiquitously in the environment, in feed and in the gastrointestinal tract of animals and humans [5,6]. It has been suggested that alpha toxin production is an essential virulence factor in the pathogenesis of NE [7], but recently it was established that only strains producing NetB toxin, a β-pore-forming toxin, are capable of inducing NE in broiler chickens under specific conditions that predispose to the disease [8,9].

Acute NE is characterized by a sudden increase in mortality, often without premonitory symptoms. Nowadays, the subclinical form is becoming more prevalent, and is mainly characterized by intestinal mucosal damage without clinical signs or mortality. This leads to a decreased digestion and absorption of nutrients, a reduced weight gain and an impaired feed conversion rate [4,8].
Table 1. Experimental groups and impact of DON on the number of chickens affected by necrotic enteritis (NE) and bodyweight (BW) gain.

| GROUP | DON (1) alone | Cp (1) alone | Cp(1) + DON (1) | DON (1) + Cp (1) | negative control |
|-------|---------------|--------------|----------------|-----------------|-----------------|
| day 1–7 | 20 ± 2.6 | 20 ± 2.6 | 20 ± 2.6 | 20 ± 2.6 | 20 ± 2.6 |
| day 8–14 | 38 ± 7 | 37 ± 4 | 47 ± 3.0 | 47 ± 3.0 | 47 ± 3.0 |
| day 15–euthanasia | 19 ± 2 | 18 ± 3 | 20 ± 6 | 20 ± 6 | 20 ± 6 |

C. perfringens challenge strain 56. The solid bars represent the average lesion score in each experimental group consisted of 3 cages of 30 chickens. After a feeding period of 3 weeks chickens were euthanized. Error bars represent SEM. Intestinal lesions in the small intestine (duodenum to ileum) were scored as previously described [7]; 0 no gross lesions; 2 small focal necrosis or ulceration (one to five foci); 3 focal necrosis or ulceration (six to 15 foci); 4 focal necrosis or ulceration (16 or more foci); 5 patches of necrosis 2 to 3 cm long; 6 diffuse necrosis typical field cases. The score 1 used for congested intestinal mucosa was not applied here because of difficulties in scoring this phenomenon objectively, and due to the lack of scientific documentation of an association between “congested intestinal mucosa” and necrotic enteritis. Birds with lesion scores of 2 or more were classified as NE positive.

doi:10.1371/journal.pone.0108775.g001

Notwithstanding the role of *C. perfringens* in poultry production losses, the mere presence of virulent strains in the intestinal tract of broilers, or even the inoculation of chickens with high doses of these strains, does not always lead to the development of NE. Predisposing factors including dietary, husbandry and immune factors [7,10,11], are required to reproduce the disease [12–14]. The best-known predisposing factor is mucosal damage caused by coccidial pathogens [13,15], which could provide *C. perfringens* with essential nutrients and thus stimulate massive overgrowth [16,17]. *C. perfringens* lacks many genes of the orthologous enzymes required for amino acid biosynthesis, among others for arginine, phenylalanine, tryptophan, tyrosine, histidine, leucine, isoleucine, valine, glutamate, lysine, methionine, serine and threonine. Therefore, *C. perfringens* growth is restricted in an environment where the amino acid supply is limited [16,18,19].

The mycotoxin deoxynivalenol (DON) is one of the most common contaminants in poultry feed worldwide. DON is a type B trichothecene produced by among others *Fusarium* (*F.* *graminearum* and *F. culmorum*). Recent data on global mycotoxin occurrence showed that 59% of 5,819 samples of animal feed tested positive for the presence of DON. The average contamination level was 1,104 μg DON/kg feed, with a maximum observed level of 49,307 μg/kg [20]. The European maximum guidance level for poultry feed is set at 5,000 μg/kg feed [21].

Poultry is considered rather tolerant to DON. It has been suggested that concentrations higher than 5,000 μg/kg feed are necessary to negatively influence the growth performance of broilers [22,23]. This mycotoxin acts as an inhibitor of the protein synthesis at the ribosomal level whereby rapidly proliferating cells in tissues with high protein turnover rates, such as the immune system and small intestine, are most affected [23]. Accordingly, DON negatively influences small intestinal epithelial cell integrity and morphology [24–29]. As a consequence of the negative effect of DON on the gastro-intestinal epithelial cells, feeding DON-contaminated diets can lead to greater susceptibility to enteric infections [27]. Only few studies have investigated the interaction...
between DON and enteric pathogens. In pigs, it has been shown in an intestinal ileal loop model that co-exposure to DON and Salmonella Typhimurium potentiates the inflammatory response in the gut [30]. In vitro, intestinal porcine epithelial cells (IPEC-1) show an increased translocation of a septicemic Escherichia coli (O75:K95) after DON exposure [26]. It is hitherto unclear whether the intestinal epithelial damage caused by contamination levels of DON below 5,000 μg/kg in feed, may act as an additional predisposing factor in broiler NE. We hypothesized that this intestinal damage may lead to higher protein availability for clostridial proliferation in the small intestine.

The objectives of this study were to examine whether DON at concentrations in the feed below the EU maximum guidance level predisposes for NE in broilers, and to gain insights in the mechanisms responsible for this interaction. Therefore, the effects of DON on the intestinal epithelial barrier function and on intestinal protein availability for clostridial proliferation were evaluated. Also, the direct effect of DON on in vitro bacterial proliferation, alpha toxin production and netB transcription was studied.

**Materials and Methods**

**Deoxynivalenol**

For the in vitro assessment of the impact of DON on growth and toxin production of C. perfringens, a DON stock solution of 2000 μg/mL (Fermentek, Jerusalem, Israel) was prepared in anhydrous methanol and stored at −20°C. Next, serial dilutions of DON were prepared in tryptone glucose yeast (TGY) broth medium.

For the animal trials, DON was produced in vitro from cultures of F. graminearum in accordance to the protocol described by Alpeiter et al. [31] (Romer Labs, Tulln, Austria), and was mixed into the experimental feed.

**Bacterial strains**

C. perfringens strain 56 has been used previously to induce NE in an in vivo model in broilers [6,32]. Originally this strain was isolated from the gut of a broiler chicken with severe NE lesions, and characterized as a netB toxin positive type A strain (no β2 or enterotoxin genes) as well as a producer of moderate amounts of alpha toxin in vitro [33].

In addition to strain 56, a netB toxin negative strain (C. perfringens strain 6 [33]) was included as negative control for in vitro netB transcription measurement.

**Birds and housing.** Non-vaccinated Ross 308 broilers were used that were obtained as one-day-old chicks from a commercial hatchery. Each group consisted of approximately equal numbers of males and females. All treatment groups were housed in the same room, in cages of 1.44 m², on a litter floor. All cages were separated by solid walls to prevent direct contact between birds from different treatment groups. Before each trial, the cages were decontaminated with peracetic acid and hydrogen peroxide (Hygasept vaporizer climasept; SARL Hygasept, Sevrey, France) and a commercial anticoccidial disinfectant (Bi-OO-Cyst Coccidial Disinfectant; Biolink, York, United Kingdom).

**Table 2. Effect of DON on villus height and crypt depth measurements.**

|                | control diet | DON diet     | P    |
|----------------|--------------|--------------|------|
| mid-duodenum   |              |              |      |
| villus height  | 2,175 ± 26.8 | 2,010 ± 52.9 | 0.014 (*) |
| crypt depth    | 143 ± 4.7    | 154 ± 9.1    | 0.269 |
| villus to crypt| 16 ± 0.5     | 13 ± 1.0     | 0.073 |
| mid-jejunum    |              |              |      |
| villus height  | 894 ± 72.6   | 792 ± 61.6   | 0.303 |
| crypt depth    | 178 ± 12.3   | 150 ± 6.4    | 0.052 |
| villus to crypt| 5 ± 0.3      | 5 ± 0.4      | 0.978 |
| mid-ileum      |              |              |      |
| villus height  | 711 ± 63.8   | 689 ± 23.6   | 0.748 |
| crypt depth    | 153 ± 10.6   | 144 ± 6.7    | 0.456 |
| villus to crypt| 5 ± 0.2      | 5 ± 0.3      | 0.487 |

Analysis was based on 9 animals per treatment, and the mean of 5 to 15 measurements per segment per animal was calculated; data are presented as weighted mean ± SEM.

(*):significantly different (P<0.05).

[doi:10.1371/journal.pone.0108775.t002]
Chickens had ad libitum access to drinking water and feed and were subjected to a 23 h/1 h light/darkness programme. The animals were not fasted before euthanasia. The environmental temperature was adjusted to the changing needs of the animals according to their age (week 1: 35°C, week 2: 30°C, week 3: 25°C).

Feed. All birds were given a starter diet during the first eight days of the experiment, and subsequently a grower diet until the end of the trial. The diet was wheat:rye (43%: 7.5%) based, with soybean meal as the main protein source during the first 16 days. From day 17 onwards, the same grower diet was used with the exception that fishmeal (30%) was added as protein source instead of soybean meal. Further details of the feed composition were as previously described [32].

In the exposed groups, an artificially DON contaminated diet was fed from day 1 onward. The contaminated feed was produced by adding DON to a control diet. To test for DON concentrations in the feed, samples were taken at three different locations in the batch and subsequently pooled. All diets were analysed for the content of DON and other mycotoxins with a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry method (LC-MS/MS) [34]. The levels of DON in the different batches of control feed was below the limit of quantification. The DON contamination level in the different batches of contaminated feed varied between 2,884 ± 800 μg/kg and 4,384 ± 1,300 μg/kg feed. All other mycotoxins tested were either absent or present in low concentrations. Table S1 shows the different mycotoxins tested, their limit of detection (LOD) and limit of quantification (LOQ) and their concentration in the different feeds.

Animal experiment 1: C. perfringens experimental infection study. The trial was performed following an adapted protocol based on a previously described experimental infection model, with the modification that no coccidial challenge was administered [32]. In the trial, 360 chicks were divided into 4 experimental groups, each group consisting of 3 cages of 30 chicks. The experimental groups are described in Table 1. One group was experimentally infected with C. perfringens and received a control diet. A second group was experimentally infected with C. perfringens and received a DON contaminated diet, while a third group was fed a DON contaminated feed but did not receive C. perfringens. A fourth group was a negative control (no C. perfringens and control feed). Gumboro vaccine (Nobilis Gum-boro D78, MSD Animal Health, Brussels, Belgium) was administered in the drinking water on day 16 to all birds. Experimental infection with C. perfringens consisted of oral inoculation of the birds with 4.10^8 cfu of C. perfringens strain 56 at days 17, 18, 19 and 20. The bacteria for the animal experiment were cultured anaerobically overnight in brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) supplemented with 0.375% glucose at 37°C. The actual number of bacteria/mL was assessed by plating tenfold dilutions on Columbia agar (Oxoid) with 5% sheep blood, incubated anaerobically overnight at 37°C. Birds that were not infected with C. perfringens received a sham inoculation with BHI broth.

Figure 3. No impact of DON on in vitro growth of C. perfringens. C. perfringens strains 6 (a) and 56 (b) were grown in TGY broth medium containing 0, 0.2, 2 or 20 μg DON/mL. Samples were taken at 0, 2, 3, 4, 5, 6, 7, 8 and 24 h after inoculation with an overnight culture of C. perfringens. The number of colony forming units (cfu) per mL was determined by bacterial plating of 10-fold dilutions. Results are presented as the mean cfu/mL. There is no significant difference between the different test conditions. doi:10.1371/journal.pone.0108775.g003

Figure 4. Net8 toxin transcription is not influenced by DON. Transcription level of net8 toxin was analysed by qRT-PCR of C. perfringens strain 56 RNA samples collected from in vitro culture material in the mid (after 3 h incubation) and late logarithmic (after 6 h incubation) growth phase. C. perfringens strain 56 was grown in absence or presence (0.2, 2, 20 μg/mL) of DON. The results for the net8 gene transcription were normalized to the rpoA gene transcription. Results are presented as the mean value of three biological replicates. Error bars represent SD. There is no significant difference between the different test conditions. doi:10.1371/journal.pone.0108775.g004
On days 21, 22 and 23, each day one third of each group was euthanized and the birds were immediately submitted to necropsy. Single-blind macroscopic NE lesion scoring of the small intestine (duodenum to ileum) was performed as previously described by Keyburn et al. [7]. Birds with lesion scores of 2 or more were classified as NE positive.

In addition, contents of three small intestinal segments of 27 birds per group of the third (DON, no C. perfringens) and fourth (negative control) experimental group were collected and stored at 220°C until further use for protein analysis. The three segments were duodenum, jejunum and ileum. The duodenum was defined as the segment encompassing the duodenal loop, whereas the jejunum was defined as the segment between the end of the duodenal loop and Meckels diverticulum. The ileum comprised the distal segment starting at Meckels diverticulum and ending at the ileo-cecal junction.

Contents of these three segments were used to determine intestinal nitrogen (N) concentration by the Kjeldahl method used for feeding stuffs (ISO2005). Percentage crude protein per dry matter of the intestinal content was calculated from Kjeldahl N values, using 6.25 as conversion factor to protein level.

The bodyweight (BW) of 30 identified chickens per experimental group was measured at day 1, 7, 14 and at the day of euthanasia. Bodyweight gain was determined as the differences in BW divided by the period of time. The presence of coccidiosis was excluded by faecal oocyst count and macroscopic coccidiosis lesion scoring of the intestines [35].

Animal experiment 2: Effect of DON on villus height and transepithelial electrical resistance. Eighteen birds were divided into 2 experimental groups, each group consisting of 3 cages of 3 chicks. One group was fed a control diet, and the other group was fed a DON-contaminated diet. All birds received Gumboro vaccine on day 16 and they all received a sham inoculation with blank BHI broth on day 17, 18, 19 and 20. On day 21, immediately after euthanasia of the animals, 1 cm samples from the mid-duodenum, mid-jejunum and mid-ileum were collected for evaluation of the intestinal morphology. These samples were fixed in neutral-buffered formalin, and processed afterwards using standard protocols for hematoxylin and eosin staining of paraffin sections. Villus height and crypt depth were measured using a light microscope with Leica LAS software (Leica Microsystems, Diegem, Belgium). The average of 5 to 15 measurements per segment per animal was calculated.

The remainder of the mid-duodenal segment was immersed into oxygenated (O2/CO2, 95/5%) Krebs Henseleit buffer solution (Sigma-Aldrich) of pH 7.4. Before opening the intestinal segment, the underlying serosal layer was stripped off. Segments were opened along the mesenteric border and rinsed with buffer solution. Per chicken, three duodenal segments of 2 cm in length were cut and each mounted in an Ussing chamber (Mussler Scientific Instruments, Aachen, Germany). Epithelial sheets had an exposed surface area of 0.28 cm². Mucosal and serosal compartments were simultaneously filled with 7 mL Krebs Henseleit buffer. Four Ag/AgCl electrodes were connected to each chamber by 3M KCl-agar bridges. The electrodes were coupled to an external six-channel microcomputer controlled voltage/current clamp. After an equilibration period of 30 minutes, the transepithelial potential difference (PD, mV) and transepithelial electrical resistance (Rt - TEER, Ω·cm²) were monitored as measures of tissue viability and integrity, respectively, with the tissue unclamped in open circuit mode. Current (Isc, µA/cm²) was calculated from Ohm’s law using the following equation Isc = Pd/Rt [36].

The in vivo experimental protocols and care of the animals were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2011/169 EC 2012/074).

Figure 5. Deoxynivalenol predisposes for C. perfringens induced necrotic enteritis. DON decreased villus height and reduced transepithelial electrical resistance (1), leading to a decreased absorption and digestion of dietary nutrients; and an increased intestinal barrier permeability, respectively. Taken together with an increased intestinal protein level, these results suggest an impaired nutrient uptake (2) and leakage of plasma amino acids (3) into the intestinal lumen, providing the necessary growth substrate for C. perfringens proliferation (4). Proliferation of virulent (netB positive) C. perfringens induces necrotic enteritis (5).

doi:10.1371/journal.pone.0108775.g005
In vitro study of the effect of DON on C. perfringens growth, alpha toxin production and netB transcription

Following concentrations of DON were tested for its effect on C. perfringens growth, alpha toxin production and netB transcription: 0, 0.2, 2 or 20 μg DON/mL TGY medium.

C. perfringens strains 6 and 56 were grown for 24 h in TGY broth medium. Subsequently, this bacterial culture was 1:1000 diluted in the different DON concentrations and incubated anaerobically at 37°C. Clostridial growth curve was assessed by bacterial plating of a ten-fold dilution series at 0, 2, 3, 4, 5, 6, 7, 8 and 24 h after inoculation. Ten-fold dilutions were made in phosphate buffered saline (PBS) solution. Six droplets of 20 μL of each dilution were plated on Columbia agar with 5% sheep blood. After anaerobic incubation overnight at 37°C, the number of colony forming units (cfu)/mL was determined by counting the number of bacterial colonies for the appropriate dilution.

Quantitative detection of alpha toxin in the C. perfringens (strain 56) culture supernatants was performed as previously described by Gholamian-dehkordi et al., using the Bio-X Alpha Toxin Elisa Kit (Bio-X Diagnostics, Jemelle, Belgium) [33]. Positive (pure alpha toxin) and negative controls (incubation buffer) were included. All tests were performed in triplicate with two technical repeats in each experiment. Subsequently, the mean optical density (OD) value was calculated relative to the positive control value, which was set at 1.

The impact of DON on netB transcription was tested by qRT-PCR [37]. The transcription levels of netB in the presence of DON were compared to non-DON contaminated test conditions normalized to the housekeeping gene rpoA4, encoding RNA polymerase subunit A. One mL of mid and late logarithmic growth phase was collected for all test conditions, as described above, from three biological replicates. Based on the growth curve, mid and late logarithmic growth phase were defined after 3 h and 6 h incubation, respectively. Cells were collected by centrifugation at 9,300×g for 5 min at 4°C. Total RNA was isolated using RNAzol RT (Sigma-Aldrich, Bornem, Belgium) and 40 ng of RNA was converted to cDNA with iScript cDNA Synthesis Kit (Bio-rad, Nazareth Eke, Belgium) in accordance with the manufacturer’s instructions. RT-qPCR was performed using SYBR-green 2x master mix (Bioline, Brussels, Belgium) with a Bio-Rad CFX-384 system. Each reaction was done in triplicate in a 12 μL total reaction mixture using 2 μL of the cDNA sample and 0.5 μM final qPCR primer concentration (Table S2). The q-PCR conditions used were 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and stepwise increase of the temperature from 65°C to 95°C (at 10s/0.5°C). Melting curve data were analysed to confirm the specificity of the reaction. For construction of the standard curve, the PCR product was generated using the standard PCR primers listed in Table S2 and DNA from C. perfringens. After purification (MSB Spin PCRapace, Stratec Molecular, Berlin, Germany) and determination of the DNA concentration with a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), the concentration of the linear dsDNA standard was adjusted to 1×10^6 to 1×10^7 copies per μL with each step differing by tenfold. The copy numbers of samples were determined by reading off the standard series with the Ct values of the samples.

Statistical analyses

Statistical program SPSS version 21 was used for data analysis. All in vitro experiments were conducted in triplicate with three repeats per experiment, unless otherwise noted. To compare the number of NE positive birds (lesion score ≥2) between different groups, binomial logistic regression was used. Bodyweight gain was analysed by using an univariate general linear model. Total protein levels, electrophysiological parameters, villus height and crypt depth measurements, in vitro assessment of clostridial growth and toxin production, were assessed by independent t-test, after determination of normality and variance of homogeneity. Significance level was set at 0.05.

Results

Animal experiments

DON significantly increases the number of chickens affected by NE. The DON-contaminated diet led to a significantly increased number of chickens with NE; i.e. 20±2.6% of the chickens in the group inoculated with C. perfringens and fed a control diet were positive for NE lesions, while in the group inoculated with C. perfringens and fed a DON-contaminated diet 47±3.0% of the broilers were positive (P<0.001) (Figure 1, Table 1). No animals with NE lesions were detected in the groups without bacterial challenge. Lesion scores of individual broiler chickens challenged with C. perfringens are shown in Figure 1. In NE positive chickens the lesions were mainly observed in the duodenum (29±0.1% and 31±0.1% of the NE positive chickens in the control and DON group, respectively) and jejunum (94±0.1% and 96±0.1%, respectively). In the ileum, only one animal in the control group and no animals in the DON group showed lesions (score 2). No statistically significant differences were observed in BW gain between the different groups (Table 1). No coccidiosis challenge was observed, since Eimeria oocysts were absent in the excreta and no macroscopic coccidiosis lesions were observed.

DON increases the intestinal protein concentration. The total protein concentration in duodenal intestinal content was significantly higher in chickens fed the DON contaminated diet (P=0.023). However, no effect of DON on the total protein concentration in jejunal and ileal intestinal content was detected (Figure 2).

DON reduces transepithelial electrical resistance in duodenal segments. No difference was observed between the control and the DON group for PD (P=0.073) and for the crypt to villus ratio in the duodenum (P=0.052). A trend was observed for reduction in the villus height to crypt depth ratio in the duodenum (P=0.014) and for the crypt depth in the jejunum in the DON group (P=0.052).

In vitro experiment

No impact of DON on C. perfringens growth, alpha toxin production and netB transcription. The results of the C. perfringens growth assay showed no influence of 0, 0.2, 2 or 20 μg DON/mL on the bacterial growth curve (Figure 3 a, b). Quantification of alpha toxin also revealed no impact of these concentrations of mycotoxin. The mean OD of the alpha toxin detection, relative to the positive control value was 1.1±0.05, 1.1±0.01, 1.1±0.03 and 1.1±0.03 in the presence of 0, 0.2, 2 or 20 μg DON/mL, respectively. Measurement of the C. perfringens transcription level of netB by qRT-PCR showed no influence of DON (Figure 4).
**Discussion**

Our data demonstrate that the mycotoxin DON is a predisposing factor for the development of NE in broiler chickens. Indeed, contamination of the diet with DON at concentrations below the EU maximum guidance level of 5,000 µg/kg feed, significantly increased the number of chickens affected with NE.

The distribution of NE lesions in the present infection study, mainly in duodenum and jejunum, is similar as in a previously described NE infection trial, where coccidiosis was included as predisposing factor [32]. The proximal part of the intestinal tract is the main absorption site for DON [22,28,38]. Proximal intestinal epithelial cells are thus exposed to high concentrations of DON following ingestion of DON-contaminated feed, and are as such sensitive due to their high protein turnover [22,39,40]. DON negatively affected the proximal part of the intestinal tract, demonstrated by the significantly reduced villus height in the duodenum. These results are in accordance with those observed by Awad et al. [28], who tested a similar contamination level and duration of exposure of DON. The decreased villus height will compromise the effectiveness of nutrient absorption due to the decreased absorption surface area [27]. Enterocytes must differentiate during their migration along the crypt-villus axis to fully express their digestive functions [41]. The sucrase and maltase activities increase for example towards the villus tip in chicks [42]. As such, the negative impact of DON on the villus height can be associated with an impaired nutrient digestion due to a reduced number of differentiated epithelial cells [27].

DON also modulates the intestinal paracellular transport leading to an increased passage of macromolecules and bacteria [26]. The intestinal barrier function is maintained by intercellular structures, including tight junctions, adherence junctions and desmosomes [25,40]. The TEER is considered as an indicator of the epithelial integrity and thus of the organization of tight junctions. In accordance with literature [26,39], we demonstrated a reduction of the TEER of the duodenal epithelium after DON exposure. These toxic effects on epithelial cells contribute to an increased protein availability in the intestinal lumen due to leakage of plasma amino acids or proteins into the gut. Consequently, this creates an environment that favors massive overgrowth of *Clostridium perfringens*. Indeed, in this study, the total duodenal protein level showed that DON has a cytotoxic effect on enterocytes, leading to an altered intestinal barrier function, resulting in an increased permeability of the intestinal wall. Additionally, the shortened villus height could lead to a decreased absorption of dietary proteins, resulting in an increased protein concentration in the intestinal lumen. These mechanisms lead to an increased protein content in the intestinal lumen, which is available for clostridial proliferation resulting in the development of necrotic enteritis.

**Supporting Information**

**Table S1** Different mycotoxins tested, their limit of detection (LOD) and limit of quantification (LOQ) and their concentration in the different feeds.

**Table S2** Primer sequences used for qRT-PCR transcriptions analysis of netB toxin. Sequences are presented from 5' to 3'.

**Acknowledgments**

The technical assistance of Mylle J, De Rycke H., Detavernier C., Puttevils C., Ameye D., Van Hecke M., De Bock Y. was gratefully appreciated. Delezie E. of the Institute for Agricultural and Fisheries Research (Melle, Belgium) is acknowledged for formulation of the feed. The authors gratefully appreciated the excellent assistance of many PhD students from the Department of Pharmacology, Toxicology and Biochemistry and the Department of Bacteriology, Pathology and Avian Diseases.

**Author Contributions**

Conceived and designed the experiments: GA FVI FP RD AM SC. Performed the experiments: GA LT MV VE. Analyzed the data: GA FVI FP RD FH LF GJ AM SC. Contributed reagents/materials/analysis tools: GJ ME SDS. Contributed to the writing of the manuscript: GA FVI FP RD AM SC. Provided deoxynivalenol for in vivo experiments: SH.

**References**

1. van der Shuis W (2000) *Clostridium perfringens* - a syndrome emerging world wide. World Poult 16: 56–57.
2. van der Shuis W (2000) Clostridial enteritis is an often underestimated problem. World Poult 16: 42–43.
3. Craven S, Stern N, Bailey J, Cox N (2001) Incidence of *Clostridium perfringens* in broiler chickens and their environment during production and processing. Avian Dis 45: 887–896.
4. Van Immerseel F, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, et al. (2004) *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol 33: 537–549.
5. Barbara AJ, Trihã HT, Glock RD, Glenn Songer J (2000) Necrotic enteritis producing strains of *Clostridium perfringens* displace non-necrotic enteritis strains from the gut of chicks. Vet Microbiol 126: 377–382.
6. Timbermont L, Lanckriet A, Gholaami-dehkordi AR, Pasmans F, Martel A, et al. (2009) Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers. Comp Immunol Microbiol Infect Dis 32: 503–512.
7. Keyburn AL, Sheedy SA, Ford ME, Williamson MM, Awad MM, et al. (2006) Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. Infect Immun 74: 6496–6500.
8. Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F (2011) Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol 40: 341–347.
9. Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, et al. (2008) NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog 4: e26.
Craven S (2000) Colonization of the intestinal tract by *Clostridium perfringens* and fecal shedding in diet-stressed and unstressed broiler chickens. Poult Sci 79: 843–849.

Cooper KK, Songer JG (2009) Necrotic enteritis in chickens: A paradigm of enteric infection by *Clostridium perfringens* type A. Anaerobe 15: 55–60.

McReynolds J, Byrd J, Anderson R, Moore R, Edrington T, et al. (2004) Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poult Sci 83: 1948–1952.

Williams R (2005) Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathol 34: 159–180.

Lee KW, Lillehei H, Jeong W, Jeoung H-Y, An D-J (2011) Avian necrotic enteritis: Experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poult Sci 90: 1381–1390.

Collier C, Holfacre C, Payne A, Anderson D, Kaiser P, et al. (2008) Coccidial-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet Immunol Immunopathol 122: 104–113.

Boyd MJ, Logan MA, Tytell AA (1948) The growth requirements of *Clostridium perfringens*. *J Gen Microbiol* 16: 317–329.

Rodrigues I, Naehler K (2012) A Three-Year Survey on the Worldwide Occurrence of Mycotoxins in Feedstuffs and Feed. Toxins 4: 663–675.

European Commission (2006) Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and deoxynivalenol contaminated wheat containing deoxynivalenol alters the gene expression in the jejunal mucosa of broiler chickens, but addition of an adsorbing agent shifts the effects to the distal parts of the small intestine. PLoS one 8: e90014.

Oselarre A, Santos R, Hauketveit V, De Backer P, Chiers K, et al. (2013) Deoxynivalenol impairs hepatic and intestinal gene expression of selected oxidative stress, tight junction and inflammation proteins in broiler chickens, but addition of an adsorbing agent shifts the effects to the distal parts of the small intestine. PLoS one 8: e23871.

Alt Peter, Posselt UK (1994) Production of high quantities of 3-acetyldeoxynivalenol and deoxynivalenol. *Appl Microbiol Biotechnol* 41: 384–387.

Gheltamianekhojiri AR, Timbermont L, Lanckriet A, Van Den Broeck W, Pedersen K, et al. (2007) Quantification of gut lesions in a subclinical necrotic enteritis model. Avian Pathol 36: 375–382.

Gheltamianekhojiri AR, Durante D, Hennings M, Hessebrock F, Van Immerseel F (2006) Molecular and phenotypical characterization of *Clostridium perfringens* isolates from poultry flocks with different disease status. Vet Microbiol 113: 143–152.

Monbaliu S, Van Poucke C, Detavernier C, Dumontin F, Van De Velde M, et al. (2009) Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-M/S method. *J Agric Food Chem* 58: 66–71.

Johnson J, Reid WM (1970) Anticoccidial Drugs - Lesion Scoring Techniques in Battery and Floor-Pen Experiments with Chickens. *Exp Parasitol* 28: 30–36.

Neirnicks E, Vervaelet C, Michels J, De Smet S, Van Den Broeck W, et al. (2011) Feasibility of the Using chamber technique for the determination of in vitro jejunal permeability of passively absorbed compounds in different avian species. *J Vet Pharmacol Ther* 34: 290–297.

Cheung JR, Keyburn AL, Carter GP, Lanckriet AL, Van Immerseel F, et al. (2010) The ViaSR two-component signal transduction system regulates NetB toxin production in *Clostridium perfringens*. Infect Immun 78: 3064–3072.

Avantaggiato G, Robert H, Angelo V (2004) Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an in vitro gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. *Food Chem Toxicol* 42: 817–824.

Marascia M, Mahfoud R, Garrny N, Fantini J (2002) The mycotoxin deoxynivalenol affects nutrient absorption in human intestinal epithelial cells. *J Nutr* 132: 2723–2731.

Bouhet S, Oswald IP (2005) The effects of mycotoxins, fungal food contaminants, on the intestinal epithelial cell-derived immune response. *Vet Immunol Immunopathol* 108: 199–209.

Applegate T, Schatzmayr G, Pricket K, Trosche C, Jiang Z (2009) Effect of aflatoxin culture on intestinal function and nutrient loss in laying hens. Poult Sci 88: 1215–1221.

Uzi Z, Platin R, Sikan D (1998) Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. *J Comp Physiol B* 168: 241–247.

Awad WA, Rehman H, Bohm J, Razzazi-Fazeli E, Zentek J (2005) Effects of luminal deoxynivalenol and L-proline on electrophysiological parameters in the jejunal mucosa of laying hens. Poult Sci 84: 928–932.

Dietrich B, Neumenschwander S, Bucher B, Wenk C (2011) *Fusarium* mycotoxin-contaminated wheat containing deoxynivalenol alters the gene expression in the liver and the jejunum of broilers. *Animal* 6: 278–291.