The Mycotoxin Deoxynivalenol Potentiates Intestinal Inflammation by *Salmonella* Typhimurium in Porcine Ileal Loops

Virginie Vandenbroucke1*, Siska Croubels1, An Martel2, Elin Verbrugghe2, Joline Goossens1, Kim Van Deun2, Filip Boyen2, Arthur Thompson3, Neil Shearer3, Patrick De Backer1, Freddy Haesebrouck2, Frank Pasmans2

1 Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, 2 Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, 3 Institute of Food Research, Norwich Research Park, Norwich, United Kingdom

Abstract

**Background and Aims:** Both deoxynivalenol (DON) and nontyphoidal salmonellosis are emerging threats with possible hazardous effects on both human and animal health. The objective of this study was to examine whether DON at low but relevant concentrations interacts with the intestinal inflammation induced by *Salmonella Typhimurium*.

**Methodology:** By using a porcine intestinal ileal loop model, we investigated whether intake of low concentrations of DON interacts with the early intestinal inflammatory response induced by *Salmonella Typhimurium*.

**Results:** A significant higher expression of IL-12 and TNFα and a clear potentiation of the expression of IL-1β, IL-8, MCP-1 and IL-6 was seen in loops co-exposed to 1 μg/mL of DON and *Salmonella Typhimurium* alone. This potentiation coincided with a significantly enhanced *Salmonella* invasion in and translocation over the intestinal epithelial IPEC-J2 cells, exposed to non-cytotoxic concentrations of DON for 24 h. Exposure of *Salmonella Typhimurium* to 0.250 μg/mL of DON affected the bacterial gene expression level of a limited number of genes, however none of these expression changes seemed to give an explanation for the increased invasion and translocation of *Salmonella Typhimurium* and the potentiated inflammatory response in combination with DON.

**Conclusion:** These data imply that the intake of low and relevant concentrations of DON renders the intestinal epithelium more susceptible to *Salmonella Typhimurium* with a subsequent potentiation of the inflammatory response in the gut.

Introduction

The contamination of food and feed with mycotoxins poses a worldwide problem with an acknowledged negative effect on both human and animal health and significant economic and international trade implications. Deoxynivalenol (DON) is a trichothecene mycotoxin frequently contaminating maize and small grain cereals in temperate regions of Europe, North America and Asia [1]. The intake of DON contaminated food or feed can lead to adverse health effects in both humans and animals. For humans, a large-scale European study on the occurrence of *Fusarium* toxins and the dietary intake showed that 57% of the tested cereals samples such as wheat were positive for DON and based on the intake estimates, it is clear that the presence of trichothecenes can pose a public health concern [2,3]. A study on the prevalence and level of urinary DON in the United Kingdom population indicated that urinary levels of DON were positively correlated with cereal intake suggesting that the European Union recommended maximum tolerable daily intake of 1 μg DON/kg BW may be exceeded. Urinary DON levels can be a valuable tool as exposure biomarker for biomonitoring in etiological studies of DON and human disease risks [4]. For human food products, the European Union sets maximum limits for DON in cereals and cereal-based products, ranging from 200 to 1750 μg/kg [5]. Anorexia, altered feed intake, reduced weight gain and immunologic alterations are associated with chronic low-doses ingestion of DON whereas acute high-dose exposure is characterized by emesis, diarrhea, vomiting and rectal bleeding. Among farm animals, pigs are considered particularly sensitive to the dietary intake of DON resulting in substantial economical losses [6].

Nontyphoidal *Salmonella* represents an important human and animal pathogen worldwide. Each year approximate 93.8 million human cases of gastroenteritis occur due to *Salmonella Typhimurium* around the world [7]. In 2009, *Salmonella* was the most
commonly reported bacteriological agent of human food borne diseases in the USA, causing approximate 44% of confirmed food borne bacterial infections [8]. *Salmonella* Typhimurium is, together with *Salmonella* Enteritidis, the most common serotype associated with human nontyphoidal salmonellosis in Europe and USA [9,10]. In pigs, clinical salmonellosis is not a common problem as *Salmonella* infections are mostly subclinical. However, these carrier pigs are an important contaminating source for the environment, other pigs and carcasses in the slaughterhouse and as such they pose a serious public health problem being the most important reservoir of *Salmonella* Typhimurium for humans [11–13].

Several studies already described the direct effects of the intake of DON contaminated food or feed on the gastrointestinal tract [14–23], however only few examined the possible link between DON ingestion and intestinal inflammation using *in vitro* intestinal cells line models [24–29]. The general effects of mycotoxins on the local intestinal immune response were reviewed by Bouhet and Oswald [24], whereas Mbandi and Pestka [27] examined the capacity of DON to potentiate chemokine and proinflammatory cytokine production in murine macrophages induced by killed irradiated suspensions of *Salmonella* Typhimurium, concluding that the induction of IL-1β, IL-6, TNFα and MIP-2 by the pathogen was potentiated by DON. Maresca et al. [26] indicated that besides the direct pro-inflammatory effect and potentiation of an existing inflammation, DON could also cause intestinal inflammation indirectly through alteration of the intestinal barrier function. Van De Walle et al. [28,29] suggested that high concentrations of DON could trigger intestinal inflammation and worsen inflammation related parameters.

Recently a potential link between food-associated exposure to certain mycotoxins including DON, and the induction and/or persistence of Inflammatory Bowel Disease (IBD) in genetically predisposed patients has been suggested [25]. At realistic doses, mycotoxins are able to cause immune and intestinal alterations comparable with those involved in human chronic intestinal inflammatory diseases [24]. In addition, infections with enteric pathogens such as non-typhoidal *Salmonella* have been implicated in the etiology of IBD [30,31].

With DON and salmonellosis being emerging issues with possible deleterious consequences for both animal and human health and with the gastrointestinal tract being the primary target for both, we aimed to test whether DON at low but relevant concentrations interacts with the intestinal inflammation induced by *Salmonella* Typhimurium using a porcine model of infection, representative of the human gastrointestinal gut [32–34].

**Materials and Methods**

**Ethics statement**

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 2010/065, May 3, 2010).

**Chemicals**

DON stock solution of 1 mg/mL (Sigma-Aldrich, Steinheim, Germany) was prepared in anhydrous methanol and stored at −20°C. Serial dilutions of DON were prepared in cell medium or Luria-Broth (LB, Sigma-Aldrich), depending on the experiment.

**Bacterial strains and growth conditions**

*Salmonella enterica* subspecies enterica serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a was used as wild type strain. To obtain highly invasive late logarithmic cultures for invasion assays, 2 µL of a stationary phase culture was inoculated in 5 mL LB and grown for 5 h at 37°C without aeration. The inocula for the intestinal loop model were prepared according to the temperature shift method for *Salmonella*. Cultures in LB were shaken at 130 rpm for 24 h at 25°C. After diluting threefold and adjusting the OD_{600 nm} to 1, the bacteria were incubated for 2 h at 37°C, with shaking at 130 rpm. The actual number of bacteria/ml was assessed by plating tenfold dilutions on Brilliant Green agar (BGA, Oxoid, Hampshire, UK).

**Cell cultures**

The IPEC-J2 cell line, a non-transformed intestinal cell line, which was originally derived from jejunal epithelium of an unsucked piglet was used for all the experiments [35,36]. IPEC-J2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM/Ham’s F-12 (1:1) (Invitrogen™, Life Technologies, Carlsbad, CA, USA) supplemented with 5% (v/v) FCS (HyClone, Cramlington, England, UK), 1% (v/v) insulin/transferrin/Naselenite (Gibco, Life Technologies, Paisley, Scotland), 1% (v/v) penicillin/streptomycin (Gibco) and 1% (v/v) kanamycin (Gibco).

**Cytotoxicity assay**

IPEC-J2 cells were seeded into a 96-well plate at a concentration of 1×10^4 cells/mL and cultured for 1 day or 21 days, representing undifferentiated proliferating and highly differentiated IPEC-J2 cells respectively, after which the cells were exposed to DON at a concentration of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 5 or 10 µg/mL during 24 h. The cells were maintained in an atmosphere of 3% CO2 at 37°C. To assess cytotoxicity, 150 µL of freshly prepared neutral red solution (33 mg/L in DMEM without phenol red) was added to 57°C for an additional 2 hours. The cells were then washed two times with Hank’s Buffered Saline Solution (HBSS) and 150 µL of extracting solution (ethanol/Mili-Q water/acetic acid, 50/49/1 (v/v/v)) was added in each well. The plate was shaken for 10 min. The absorbance was determined at 540 nm using a microplate ELISA reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland). The percentage of viable cells was calculated using the following formula:

\[
\text{% viability} = 100 \times \frac{(a-b)}{(c-b)}
\]

In this formula, a = OD_{540 nm} derived from the wells incubated with DON, b = OD_{540 nm} derived from blank wells, c = OD_{540 nm} derived from untreated control wells.

**Invasion and translocation assays of *Salmonella* Typhimurium in porcine intestinal epithelial cells**

To examine whether the ability of *Salmonella* Typhimurium to invade in and pass over a monolayer of IPEC-J2 cells was altered after pre-exposure of the IPEC-J2 cells to DON, invasion and translocation assays were performed.

For the invasion assays, IPEC-J2 cells were seeded in 24-well plates at a density of 1×10^5 cells per well and were cultured for 1 day or 21 days depending on the experimental setup. The 1-day-old cells were then exposed either to non-cytotoxic concentrations of DON up to 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 5 or 10 µg/mL, and to 0.25 µg/mL which decreased the percentage of viable cells to 62.3% as determined by the cytotoxicity assay described above. The 21-days-old IPEC-J2 cells were exposed to non-cytotoxic concentrations of DON up to 1 µg/mL. The invasion assay was performed as described by Boyen et al. [37]. To quantify the transepithelial passage of...
Salmonella Typhimurium over IPEC-J2 cells exposed to DON, IPEC-J2 cells were seeded on Transwell® inserts with a pore size of 3.0 μm and membrane diameter of 6.5 mm (Corning Costar Corp., Cambridge, MA) at a density of 5×10^5 cells/mL and cultured until 21 days after reaching confluence. Cell medium was refreshed every 3 days. DON was added at concentrations of 0.1, 0.25, 0.5, 0.75 or 1 μg/mL to the apical side in 200 μL of culture medium. The basolateral side received 1 mL of blank culture medium. After 24 h of treatment with DON, the Transwell® inserts were washed three times with HBSS. Then *Salmonella Typhimurium* was added to the apical compartment at a concentration of 2×10^7 Colony Forming Units (CFU/mL) suspended in IPEC-J2 medium with the respective concentrations of DON but without supplementation of antibiotics. The basolateral compartment was filled with antibiotic-free IPEC-J2 medium. After 60 min at 37°C and 5% CO_2, the number of bacteria (CFU/mL) was determined in the basolateral compartment by plating 10-fold dilutions on BGA plates. Transepithelial electrical resistance (TEER) measurements were performed before and after the incubation with DON in order to evaluate the cell barrier integrity.

**Porcine intestinal ileal loop experiments**

Considering the fact that the gastrointestinal tract and the immune system of pigs are very similar to that of humans [32–34], pig intestinal loops were used to reproduce *Salmonella Typhimurium* induced intestinal inflammation.

Two 5-week-old pigs were used in the gut loop experiments. Feed was deprived for 16 h before surgery. The pigs were anesthetized with a mixture of tiletamine+zolazepam (Zoletil 100, Virbac, Carros, France) and xylazine (Xyl-M 2%, VMD, Arendonk, Belgium), given intramuscularly at 0.2 mL/kg bodyweight after which they were intubated intratracheally. Anesthesia was maintained with 1–3% isoflurane in conjunction with 1% pure oxygen using a semiclosed circuit. Intramuscular injection of fentanyl (5 μg/kg BW/h) was used for pain management. The porcine ligated loop model was performed as described before with slight modifications [37]. After cleaning and disinfection of the abdomen, a ventral line laparotomy was performed aseptically and twelve loops were produced commencing at the distal ileum, each 6 cm in length with a short intervening segment in between. Ligation was done by an intestinal circumferential ligature through the mesentery without damaging grossly visible mesenteric arcades and thus maintaining full blood supply for both loops and inter-loop segments. A volume of 1 mL of each test condition was injected into each loop. In each pig, the treatments were randomly assigned and performed in triplicate. Following test conditions were included: negative control (LB), 1 μg/mL of DON in LB, 4×10^6 CFU/mL of *Salmonella Typhimurium* and 1 μg/mL of DON in combination with 4×10^6 CFU/mL of *Salmonella Typhimurium*. Piglets were maintained under anesthesia for 6 h after which they were euthanized using T61 intravenously (0.3 mL/kg bodyweight) (Intervet, Ukkel, Belgium) while still anesthetized. The pieces of the ligated intestine were then quickly excised from each loop and processed for RNA isolation.

**Analysis of intestinal cytokine response**

For RNA extraction pieces of tissue samples were collected from the ileal loops, immediately frozen in liquid nitrogen and stored at −70°C until analysis. Total RNA from the intestinal samples was isolated from cultures of *Salmonella Typhimurium*, a microarray analysis was performed on RNA isolated from cultures of *Salmonella Typhimurium* grown to logarithmic and stationary phase in the presence or absence of 0.250 μg/mL of DON. This concentration was chosen based on the results of the in vitro assays on IPEC-J2 cells.

Stationary phase cultures were obtained by aerated, overnight culture at 37°C in 5 mL LB in 50 mL flasks. To obtain highly invasive late logarithmic culture, 2 μL of a stationary phase culture was inoculated in 5 mL LB and grown for 3 hours at 37°C without aeration [42].

Of the stationary and logarithmic culture respectively, 2.00 OD_600 units were harvested and RNA was extracted and purified using SV Total RNA Isolation Kit (Promega Benelux bv, Leiden, The Netherlands) according to manufacturers’ instructions. The quality and purity of the isolated RNA was determined using a Nanodrop spectrophotometer and Experion RNA StdSens Analysis kit (Biorad). The SALSA microarrays and protocols for RNA labeling, microarray hybridization and subsequent data acquisition have been described previously [43]. RNA (10 μg) from 3 independent biological replicates of DON treated and untreated (control) logarithmic and stationary phase cultures was labeled with Cy5 dCTP and hybridized to SALSA microarrays with 400 ng of Cy3 dCTP labeled gDNA, as a common reference. Genes were assessed to be statistically significantly differently expressed between the DON treated and untreated controls at each growth phase by an analysis of variance test with a Benjamini
and Hochberg false discovery rate of 0.05 and with a ≥1.5-fold change in the expression level.

Micro array accession number

The microarray data discussed in this publication are MIAME compliant and have been deposited in NCBI's Gene Expression Omnibus [44] and are accessible through GEO Series accession number GSE29399 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29399).

Statistical analysis

All in vitro experiments were conducted in triplicate with three repeats per experiment, unless otherwise noted. The data were analyzed using Student's t-test to address the significance of difference between mean values with significance set at 0.05. The differences in mRNA expression among groups were assessed by performing two-factor ANOVA after determination of normality and variance homogeneity. Multiple comparisons were performed using LSD. Not normally distributed data were analyzed using the non parametric Kruskal-Wallis analysis, followed by Mann-Whitney test using SPSS 17.0 Software (SPSS Inc., Chicago, IL, USA). Significance level was set at 0.05.

Results

DON is more toxic to undifferentiated than to differentiated IPEC-J2 cells

The cytotoxic effect of DON on IPEC-J2 cells as determined using the neutral red assay is shown in Figure 1. The viability of 1-day-old IPEC-J2 cells was significantly decreased by exposure to concentrations of DON higher than 0.1 μg/mL (Figure 1A). DON concentrations up to 10 μg/mL did not significantly affect the viability of differentiated IPEC-J2 cells (Figure 1B). However, although not significant, exposure of differentiated IPEC-J2 cells to 5 and 10 μg/mL of DON during 24 h reduced the viability to 86±/−7% and 80±/−4% respectively.

DON promotes the invasion of Salmonella Typhimurium in IPEC-J2 cells

The results of the invasion assay with Salmonella Typhimurium in actively dividing IPEC-J2 cells grown are shown in Figure 2A whereas Figure 2B illustrates the invasion of Salmonella Typhimurium in differentiated IPEC-J2 cells.

The invasion of Salmonella Typhimurium was higher in undifferentiated proliferating IPEC-J2 cells exposed to concentrations of DON higher than 0.025 μg/mL in comparison to non

Table 1. List of genes and sequences of the primers used for quantitative PCR analysis.

| Gene name | Forward primer (5’→3’) | Reverse primer (3’→5’) | Accession number |
|-----------|------------------------|------------------------|-----------------|
| HPRT      | GAGCTACTGTAATGACAGTGCAACG | CCAAGTCATTATATCTTCAACAATCAA | NM_001032376 |
| HIS       | AAACAGATCTGGCGCTTC | GTCTCCAAAAGGGCAAC | NM_213930 |
| IL-1β     | GGGACCTGAAGAGAAATG | CTTTCCTTGATCCCTAAGG | NM_001005149 |
| IL-6      | CACCCGTCCTTGAGGTTT | GGGGTGGCTTGTCTGGAT | M86923 |
| IL-8      | TTCTGACGGTCTCTGTAGGCG | GGTGGACAGTGGAATGC | M86923 |
| IL-12     | CCCTTCTCTGCCTCACAAC | GTCTCCGGATATTCTTTCG | U08317 |
| IL-18     | ATGCGGTCTTTGACTTCTT | CTGACAGAGATGGTACCTG | AB010003 |
| TNFα      | CCCCCAGAGAAGAAGATTC | CCGGGCTTATCTGAGGTGTA | NM_214022 |
| IFNγ      | CATTCAAGAGAAGCATGGAT | GAGTACCTGATGGCTTGG | AY188090 |
| MCP-1     | CAGAGAGTACACAGCAGCAGCA | TCCAGGTGGCTTATGGGAT | NM_214214 |

Figure 1. Percentage viability (%) of IPEC-J2 cells exposed to different concentrations of DON (0.005–1 μg/mL). IPEC-J2 cells were grown to a confluent monolayer during 1 day (A) or grown until differentiation during 21 days (B). Twenty-four hours after incubation with DON, the cytotoxic effect was determined using neutral red assay. Results represent the means of 3 independent experiments conducted in triplicate and their standard error of the mean. * refers to a significantly higher cytotoxic effect compared to the unexposed control cells (p<0.05). doi:10.1371/journal.pone.0023871.g001
DON increases the transepithelial passage of Salmonella Typhimurium through the intestinal epithelium

We assessed the passage of Salmonella Typhimurium through 21-days-old IPEC-J2 cells treated for 24 h with non-cytotoxic concentrations of DON varying from 0.1 to 1 µg/mL (Figure 3). Exposure to DON for 24 h did not lead to a decrease in TEER (data not shown) indicating no loss of integrity of the epithelial monolayer. However, there was a significant increase in the passage of Salmonella Typhimurium bacteria (p<0.05) after exposure to 0.5, 0.75 and 1 µg/mL of DON in comparison to non treated IPEC-J2 cells.

Figure 3. Impact of DON on transepithelial passage of Salmonella Typhimurium. IPEC-J2 cells seeded onto inserts until differentiation were either exposed to blank medium or treated with different concentrations of DON (0.1; 0.25; 0.5; 0.75 or 1 µg/mL) for 24 h, prior to measuring the transepithelial passage of Salmonella Typhimurium. The results show the means of four independent experiments conducted in triplicate with their standard error of the mean. * refers to a significantly higher translocation of the bacteria compared to the unexposed control cells (p<0.05).
doi:10.1371/journal.pone.0023871.g003

DON potentiates the intestinal inflammatory response to Salmonella Typhimurium in the porcine intestinal ileal loop model

Using the porcine intestinal loop model, the effect of 1 µg/mL of DON, Salmonella Typhimurium and co-exposure of Salmonella Typhimurium with 1 µg/mL of DON on the intestinal mRNA expression levels of the cytokines (IL-1β, IL-6, IL-12, IL-10, IFNγ and TNFα) and chemokines (IL-8 and MCP-1) 6 hours post administration was examined. The results are illustrated in Figure 4. Exposure to 1 µg/mL of DON did not significantly affect the mRNA expression level of any cytokine and chemokine tested. Co-exposure of the ileal loops to 1 µg/mL of DON and Salmonella Typhimurium for 6 hours, resulted in a potentiated intestinal immune response in comparison to loops incubated with Salmonella Typhimurium but without the presence of DON. Although not significant except for TNFα and IL-12 (p<0.05), there was a higher increase in fold change in mRNA expression compared to the Salmonella Typhimurium inoculated loops for IL-1β (10.9 to 6.1 respectively), IL-12 (6.3 to 2.3 respectively), IL-8 (6.2 to 3.0 respectively), MCP-1 (2.5 to 1.2 respectively), TNFα (1.8 to 0.9 respectively) and IL-6 (1.6 to 1.1 respectively). However, no increase in mRNA expression level was seen for IL-18 and IFNγ.

Gene expression of Salmonella Typhimurium is affected by exposure to 0.250 µg/mL of DON

In the stationary phase cultures of Salmonella Typhimurium, exposure to 0.250 µg/mL of DON did not affect the gene expression level of any gene tested in the microarray analysis.

In the logarithmic culture, 159 genes were significantly (p<0.05) up-or downregulated ≥1.5-fold by exposure to 0.250 µg/mL of DON [Table S1]. A general reduction in expression of genes involved in energy production as well as an increased expression of emrC/Ab multidrug efflux systems was seen. Six genes known to play a role in the pathogenesis of a Salmonella infection were affected by exposure to 0.250 µg/mL of DON. Three Salmonella Pathogenicity Island-3 (SPI-3) genes were either upregulated (marT and fhuD) or downregulated (csgR), as well as the gene encoding for the SipA
protein and fliD and flgL, two genes encoding for hook-associate proteins, which were downregulated.

**Discussion**

Both DON and salmonellosis are worldwide emerging issues posing a threat for both animal and human health. To the best of our knowledge this is the first time that the combined effect of DON and an enteropathogenic pathogen such as *Salmonella Typhimurium* in vivo is demonstrated.

A porcine gut loop model was used to determine whether co-exposure to a physiologically relevant concentration of DON could potentiate the early intestinal immune response induced by *Salmonella Typhimurium* infection in the intestine. The choice for administering 1 mg/mL of DON was based mainly on the Commission Recommendation 2006/576/EC setting down 0.9 mg/g of DON in complementary and complete feeding stuff for pigs as guidance value [45]. Also DON concentrations in human intestine are estimated between 0.160 mg/mL and 2 mg/mL depending on the contamination level of the food [17]. Moreover, 1 mg/mL was found non-cytotoxic to differentiated IPEC-J2 cells. Undifferentiated proliferating IPEC-J2 cells are more susceptible for the toxic effects of DON compared to highly differentiated cells, as shown by the cytotoxicity assay. These results correspond with the literature describing DON to be less cytotoxic for differentiated Caco-2 cells compared to dividing cells. No cytotoxicity was observed after 24 h exposure in differentiated cells whereas IC10 values in the range 0.9–1.2 μM (0.260–0.356 mg/mL) were measured for dividing cells [46].

The results presented in this study provide evidence that DON at low but relevant intestinal concentrations enhances the intestinal inflammatory response to *Salmonella Typhimurium*. This is indicated by a significantly higher expression of IL-12 and TNFα (p<0.05) and a clear potentiation of the expression of IL-1β, IL-8, MCP-1 and IL-6 in loops co-exposed to 1 mg/mL of DON and *Salmonella Typhimurium*.

The enhanced intestinal inflammation may be due to a stimulation of *Salmonella Typhimurium* invasion in and translocation over the intestinal epithelium, as indicated by the results of the assays performed on both dividing and differentiated IPEC-J2 cells. It has indeed been suggested that bacterial invasion stimulates the innate pathways of inflammation by recognition of

![Figure 4](https://www.plosone.org/doi/10.1371/journal.pone.0023871.g004)
the pathogen-associated molecular patterns of *Salmonella* Typhi-
murium by TLRs present on epithelial cells and monocytes resulting in the production of several cytokines such as IL-8 [47].

Microarray analysis on the *Salmonella* Typhi-murium gene expression revealed that exposure to DON only changed the gene expression in logarithmic phase culture of *Salmonella* Typhi-murium and six genes of possible importance in the pathogenesis of a *Salmonella* infection were affected. The *marT* gene, a non essential gene for virulence, encodes for a regulatory protein and could be involved in other aspects of pathogenesis, such as chronic infection and host specificity [48]. The *fdiL* and *cigR* gene products do not exhibit sequence similarity to proteins with known functions in the sequence database [48]. The genes *fdiD* and *fdig* encode for proteins involved in flagellar biosyntheses [49] and the effector protein SipA has been shown to modulate actin dynamics in order to promote *Salmonella* entry into epithelial cells [50]. The general reduction in expression of genes involved in energy production indicates a possible toxic effect of DON on *Salmonella*. The increased expression of *emrAB* multidrug efflux systems suggests that these could be involved in the removal of DON out of the bacterium. No changes in gene expression were seen in the stationary phase culture of *Salmonella* Typhi-murium exposed to DON, indicating that the *Salmonella* virulence was not enhanced by DON. Since none of these bacterial gene expression alterations seem to offer a clear explanation for the increased invasion and translocation of *Salmonella* Typhi-murium and the subsequent potentiated inflammatory response seen after coexposure to DON, we can thus conclude that the action of DON is more likely to occur on the epithelial cells than on *Salmonella*. This enhanced intestinal inflammation may be of importance for patients genetically predisposed for IBD since both DON and *Salmonella* are mentioned as factors of potential etiological importance in the development of this chronic intestinal disorder [25,30,31].

The enhanced susceptibility of intestinal epithelial cells to *Salmonella* invasion and translocation is in accordance with our previous findings that concentrations of DON as low as 0.025 μg/ml enhance the uptake of *Salmonella* Typhi-murium in porcine macrophages associated with ERK1/2 induced cytoskeletal reorganization [51]. Results of our present and previous studies thus indicate that DON may influence both the intestinal and systemic phase of a *Salmonella* Typhi-murium infection, leading to increased host susceptibility to this pathogen.

In pigs, intake of DON contaminated feed might result in a higher infection level in the herd and consequently a higher public health risk for salmonellosis from the consumption of contaminated pork meat.

In conclusion, our results indicate that the intake of DON contaminated food or feed at realistic contamination levels and after short-term exposure, could render the intestinal epithelium more susceptible for invasive food pathogens such as *Salmonella* Typhi-murium with a subsequent amplification of the inflammatory processes in the gut. Considering the frequent occurrence of DON in cereal-based foods and feeds worldwide, the importance of these findings should not be underestimated.

### Supporting Information

**Table S1** Results of micro-array analysis of *Salmonella* Typhi-murium exposed to 0.250 μg/mL of deoxynivale-nol. (XLS)

### Acknowledgments

The technical assistance of Anja Van den Busche was gratefully appreciated.

### Author Contributions

Conceived and designed the experiments: VV SC FP. Performed the experiments: VV AM EV JG AT NS. Analyzed the data: VV SC KVD FB AT NS FP. Contributed reagents/materials/analysis tools: VV SC AM AT NS PDB FH FP. Wrote the paper: VV SC FP.

### References

1. Bottalico A, Perrone G (2002) Toxigenic Fusarium species and mycotoxins associated with head blight in small-grain cereals in Europe. European Journal of Plant Pathology 107(7): 611–24.

2. González-Obaya L, Cortes C, Soriano JM, Molo JC, Manes J (2011) Occurrence of deoxynivalenol and T-2 toxin in bread and pasta commercialised in Spain. Food Chem 124(1): 156–61.

3. Schothorst RC, van Egmond HP (2004) Report from SCoOP task 3.2.10 “collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states” - Subtask: trichothecenes. Toxicol Lett 153(1–3): 133–43.

4. Turner PC, Rothwell JA, White KLM, Gong Y, Cade JE, et al. (2008) Urinary deoxynivalenol is correlated with cereal intake in individuals from the United Kingdom. Environ Health Perspect 116(1): 21–5.

5. Anonymous (2006) Commission regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union.

6. Robin J (1992) The significance of the mycotoxins deoxynivalenol, zearalenone and ochratoxin A for agricultural domestic animals. Arch Tierernah 42: 95–111.

7. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, et al. (2010) The Global Burden of Nontyphoidal Salmonella Gastroenteritis. Clin Infect Dis 50(6): 802–9.

8. Prevention CIDCa (2009) Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food-10 states. MMWR Morb Mortal Wkly Rep 59(4): 418–22.

9. Eurosurveillance website International trends in *Salmonella* serotypes 1998–2003: a surveillance report from the Enter-net international surveillance network. Eurosurveillance monthly releases 9 issue 11 2004. Available: http://www.eurosurveillance.org/index2.asp. Accessed 2011 May 2.

10. Doyle E, Kaspar C, Archer J, Kiss R (2009) White paper on human illness caused by *Salmonella* from all food and non-food vectors2009. Food Research Institute website. Available: http://frinwic.edu/docs/pdf/FRI_Brief_Salmonella_human_illness_6_09.pdf. Accessed 2011 May 2.

11. Boyen F, Harsebroek E, Maes D, Van Immerseel F, Ducatele R, et al. (2008) Non-typhoidal *Salmonella* infections in pigs: A closer look at epidemiology, pathogenesis and control. Vet Mic 130(1–2): 1–19.

12. Donné E, Paans M, Boyen F, Van Immerseel F, Adrianiu C, et al. (2005) Survival of *Salmonella* serovar Typhi-murium inside porcine monocytes is associated with complement binding and suppression of the production of reactive oxygen species. Vet Mic 107(3–4): 205–14.

13. Wray CWA, ed. *Salmonella* infections in pigs. Wallingford: CAD International; 2000.

14. Diesing AK, Nossel G, Panter P, Walk N, Post A, et al. (2011) Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicol Let 200(1–2): 8–18.

15. Pintor P, Nosagyred JP, Del Rio JC, Moreno C, Marin DE, et al. (2009) The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. Toxicol Appl Pharmacol 237(1): 41–8.

16. Van De Walle J, Sergeant T, Pironi N, Toussaint O, Schneider YJ, et al. (2010) Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. Toxicol Appl Pharmacol 245(3): 291–8.

17. Sergeant T, Parysz M, Garsoù S, Passemier L, Schneider YJ, et al. (2006) Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicol Let 164(2): 167–76.

18. Danicke S, Hegewald AK, Kahlert S, Kluse J, Rothkotter HJ, et al. (2010) Studies on the toxicity of deoxynivalenol (DON), sodium metabisulfite, DON-sulfonate (DONS) and de-epoxy-DON for porcine peripheral blood mononuclear cells and the Intestinal Porcine Epithelial Cell lines IPEC-1 and IPEC-J2; and on effects of DON and DONS on piglets. Food Chemical Toxicol 48(8–9): 2154–62.

19. Kasuga F, Hara-Kudo Y, Saito N, Kumagai S, Sugita-Konishi Y (1998) In vitro effect of deoxynivalenol on the differentiation of human colonic cell lines Caco-2 and T84. Mycopathologia 142(3): 161–7.

20. Koll-Claus M, Castellote J, Joly B, Bourges-Abelha N, Raymond-Letron I, et al. (2009) Development of a pig jejunal explant culture for studying the
gastrointestinal toxicity of the mycotoxin deoxynivalenol: Histopathological analysis. Toxicol in Vitro 23(8): 1580–4.

21. Marques M, Mahfoud R, Garmy N, Fantini J (2002) The mycotoxin deoxynivalenol affects nutrient absorption in human intestinal epithelial cells. J Nutr 132(9): 2723–31.

22. Öhremski K, Zielonka I, Gajecka M, Jakimiuk E, Bakula T, et al. (2008) Histological estimation of the small intestine wall after administration of feed containing deoxynivalenol, T-2 toxin and zearalenone in the pig. Pol J Vet Sci 11(4): 339–45.

23. Pinton P, Braico C, Nougayrede JP, Lafitte J, Taranu I, et al. (2010) Deoxynivalenol impairs porcine intestinal barrier function and decreases the protein expression of claudin-4 through a mitogen-activated protein kinase-dependent mechanism. J Nutr 140(11): 1956–62.

24. Bouhet S, Oswald IP (2005) The effects of mycotoxins, fungal food contaminants, on the intestinal epithelial cell-derived innate immune response. Vet Immunol and Immunopath 108(1–2): 199–209.

25. Marques M, Fantini J (2010) Some food-associated mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. Toxicon 56(3): 282–94.

26. Marques M, Yahi N, Younes-Salek L, Boyron M, Caporiccio B, et al. (2008) Both direct and indirect effects account for the pro-inflammatory activity of enteropathogenic mycotoxins on the human intestinal epithelium: Stimulation of interleukin-8 secretion, potentiation of interleukin-1 beta effect and increase in the trans epithelial passage of commensal bacteria. Toxicol Appl Pharmacol 229(1): 84–92.

27. Mbani E, Pestka JJ (2006) Deoxynivalenol and satratoxin G potentiate proinflammatory cytokine and macrophage inhibitory protein 2 induction by Listeria and Salmonella in the macrophage. J Food Prot 69(6): 1334–9.

28. Van De Walle J, Romier B, Larondelle Y, Schneider Y-J (2008) Influence of deoxynivalenol on NF-kappaB activation and IL-8 secretion in human intestinal Caco-2 cells. Toxicol Lett 177(3): 205–14.

29. Van De Walle J, During A, Piront N, Toussaint O, Schneider Y-J (2010) Physio-pathological parameters affect the activation of inflammatory pathways by deoxynivalenol in Caco-2 cells. Toxicol in Vitro 24(7): 1890–8.

30. Gradel KO, Nielsen HL, Schonheyder HC, Ejlersen T, Kristenst B, et al. (2009) Increased Short- and Long-Term Risk of Inflammatory Bowel Disease After Salmonella or Campylobacter Gastronenteritis. Gastroenterology 137(2): 495–501.

31. Jess T, Simonsen J, Nielsen NM, Jorgensen KT, Bager P, et al. (2011) Enteric Salmonella or Campylobacter infections and the risk of inflammatory bowel disease. Gut 60(3): 318–24.

32. Niewold TA, Kerstens HHD, van der Meulen J, Smits MA, Huls MM (2005) Development of a porcine small intestinal cDNA micro-array: characterization and functional analysis of the response to enterotoxigenic E.coli. Vet Immunol Immunopath 105(3–4): 317–29.

33. Almond GW (1996) Research applications using pigs. Veterinary Clinics of North America-Food Animal Practice 12(3): 707.

34. Rothkötter HJ, Sowa E, Palsb R (2002) The pig as a model of developmental immunology. Hum Exp Toxicol 21(9–10): 533–6.

35. Rhooids JM, Chen W, Chu P, Berschneider HM, Argenzio RA, et al. (1994) L-glutamine and L-aspargine stimulate Na+-H+ exchange in porcine jejunal enterocytes. Am J Physiol 266(5): G828–G30.

36. Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, et al. (2006) Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem Cell Biol 125(3): 293–305.

37. Boyen J, Paumans F, Van Immerseel F, Donne E, Morgan E, et al. (2009) Porcine in vitro and in vivo models to assess the virulence of Salmonella enterica serovar Typhimurium for pigs. Lab Anim 43(1): 46–52.

38. Rozen S, Skeletsy HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Kravetz S, Misener S, eds. Bioinformatics Methods and Protocols: methods, Molecular Biology. Totowa: Human Press; 2000. pp 365–56.

39. Volf J, Boyen J, Fahlady M, Pavlova B, Navratilova J, et al. (2007) Cytokine response of porcine cell lines to Salmonella enterica serovar Typhimurium and its InvA and InvE mutants. Zoonoses Public Health 54: 286–93.

40. Volf J, Havlickova H, Hradecka H, Ondrackova P, Matiasovic J, et al. (2010) Epidemiology and interaction of Salmonella enterica serovar Derby, Influenza and Typhimurium with porcine alveolar macrophages. Vet Mic 146(1–2): 105–10.

41. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(Delta Delta C) method. Methods 25(4): 402–8.

42. Lundberg U, Vinatzer U, Berdthuk D, von Gabain A, Baccarini M (1999) Growth phase-regulated induction of Salmonella-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. J Bacteriol 181(11): 3433–7.

43. Nagy G, Danino V, Dobrindt U, Pallen M, Chaudhuri R, et al. (2006) Down-regulation of key virulence factors makes the Salmonella enterica serovar Typhimurium rifA mutant a promising live-attenuated vaccine candidate. Infect Immun 74(10): 5914–23.

44. Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data and repository. Nucleic Acids Res 30(1): 201–10.

45. Anonymous (2006) Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 toxin and fumonisins in products intended for animal feeding (2006/576/EC). Official Journal of the European Union.

46. Bony S, Carcelen M, Olivier I, Devaux A (2006) Genotoxicity assessment of deoxynivalenol in the Caco-2 cell line model using the Comet assay. Toxicol Lett 166(1): 67–76.

47. Tukel C, Raffatelli M, Chessa D, Wilson RP, Acker K, et al. (2006) Neutrophil influx during non-typhoidal salmonellosis: who is in the driver’s seat? FEMS Immunol Med Microbiol 46(3): 229–37.

48. Blank-Potard AB, Solomon F, Kayser J, Groisman EA (1999) The SPI-3 pathogenicity island of Salmonella enterica. J Bacteriol 181(3): 998–1004.

49. Yokoseki T, Kutsukake K, Ohnishi K, Iino T (1995) Functional analysis of the flagellar genes in the fliD operon of Salmonella Typhimurium. Microbiology-Uk 141: 1715–22.

50. Rozen S, Skeletsy HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Kravetz S, Misener S, eds. Bioinformatics Methods and Protocols: methods, Molecular Biology. Totowa: Human Press; 2000. pp 365–56.

51. Vandenbroucke V, Croubels S, Verbrugghe E, Boyen F, Pasmans F, Van Immerseel F, Donne E, Morgan E, et al. (2009) Porcine in vitro and in vivo models to assess the virulence of Salmonella enterica serovar Typhimurium for pigs. Lab Anim 43(1): 46–52.

52. Rozen S, Skeletsy HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Kravetz S, Misener S, eds. Bioinformatics Methods and Protocols: methods, Molecular Biology. Totowa: Human Press; 2000. pp 365–56.

53. Volf J, Boyen J, Fahlady M, Pavlova B, Navratilova J, et al. (2007) Cytokine response of porcine cell lines to Salmonella enterica serovar Typhimurium and its InvA and InvE mutants. Zoonoses Public Health 54: 286–93.

54. Volf J, Havlickova H, Hradecka H, Ondrackova P, Matiasovic J, et al. (2010) Epidemiology and interaction of Salmonella enterica serovar Derby, Influenza and Typhimurium with porcine alveolar macrophages. Vet Mic 146(1–2): 105–10.

55. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(Delta Delta C) method. Methods 25(4): 402–8.