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Original article

The mycotoxin deoxynivalenol promotes uptake of *Salmonella* Typhimurium in porcine macrophages, associated with ERK1/2 induced cytoskeleton reorganization

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Abstract – Both the mycotoxin deoxynivalenol (DON) and *Salmonella* Typhimurium are major issues in swine production. This study aimed at examining the interaction between DON and *Salmonella* Typhimurium at the level of the porcine innate immune system, represented by macrophages. First, we assessed the direct cytotoxic effect of DON on porcine macrophages. Incubation with 0.25 µg/mL of DON or higher resulted in a significant cytotoxic effect after 24 h of incubation. Secondly, the direct toxic effect of DON on the growth and on the expression of *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 virulence genes of *Salmonella* Typhimurium was determined. At low non-cytotoxic concentrations, as can be found in the serum of pigs, DON did not have any effect on either growth or virulence gene expression of *Salmonella* Typhimurium. However, when the invasion and intracellular survival of *Salmonella* Typhimurium in macrophages preexposed to 0.025 µg/mL of DON was examined, DON significantly promoted the uptake of *Salmonella* Typhimurium into macrophages. The enhanced uptake coincided with marked F-actin reorganization of the cells, which was due to the activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). These results suggest that low but relevant concentrations of DON modulate the innate immune system and could thus increase the susceptibility of pigs to infections with *Salmonella* Typhimurium.

deoxynivalenol / *Salmonella* Typhimurium / pig / macrophage / cytoskeleton

1. INTRODUCTION

In the northern temperate regions the mycotoxin deoxynivalenol (DON) is one of the most frequent contaminants of maize and small grain cereal [4]. This contamination of cereal crops in the field is seen under low temperature and high humidity conditions. If critical concentrations of DON in diets for farm animals are exceeded, the health, growth and reproductive performance of animals may be impaired [11]. The toxic effects of DON have been well documented and among farm animals, pigs seem to

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be particularly sensitive to the dietary intake of DON. DON has been associated with symptoms varying from partial feed refusal and decreased feed intake at feed concentrations as low as 1–2 mg/kg feed, to vomiting and complete feed refusal at concentrations of more than 20 mg/kg feed [22, 34]. Substantial economic losses have therefore been attributed to DON contamination of pig feed [2].

The mechanism of toxicity for trichothecenes, to which DON belongs, is complex, but the biochemical basis is non-competitive inhibition of different steps in the protein synthesis by interfering with peptidyl transferase at the active site on ribosomes [8]. However, many of their toxic effects might also be related to a rapidly ensuing dysregulation of intracellular cell signaling and consequent alterations in downstream gene expression [27]. Exposure to low levels of trichothecenes appears to promote expression of a diverse array of cytokines and proinflammatory genes in vitro and in vivo via a mechanism known as the ribotoxic stress response that involves multiple intracellular signaling cascades [26, 38, 39]. Trichothecenes such as DON are also known to rapidly activate mitogen-activated protein kinase (MAPK) via a process termed the "ribotoxic stress response". These MAPK modulate numerous physiological processes including cell growth, differentiation and apoptosis and are crucial for the signal transduction in the immune response. The members of the MAPK family can be classified into three subfamilies: ERK/MAPK, p38 and JNK. Yang et al. [37] described that DON induces p21 mRNA stability in terms of interaction with RNA-binding proteins extracellular signal-regulated protein kinase 1/2 (ERK1/2) activation. P21 activated kinases (PAK) are known to modulate cell morphology, actin/microtubule dynamics and cell motility [3] whereas ERK is able to phosphorylate several target proteins in the cytoplasm including cytoskeletal proteins. In contrast to these stimulatory effects, high doses of trichothecenes promote rapid onset of leucocyte apoptosis and this will be manifested as immunosuppression [26].

In European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the predominant serovar isolated from pigs which most often carry the bacterium without obvious symptoms [7, 15]. These carrier pigs, however, play an important role as a source of contamination for the environment and for other animals and after slaughter, their carcasses can be a source of contamination for other carcasses in the slaughter facility [13]. Macrophages play an important role in the pathogenesis of *Salmonella* infections in pigs as the bacteria are able to survive and even multiply intracellularly after bacterial entry into the cells. Macrophage invasion coincides with membrane ruffles, bacterium uptake and formation of *Salmonella* containing vacuole [17, 25]. Two major virulence determinants of *Salmonella* Typhimurium are the *Salmonella* pathogenicity island 1 (SPI-1) for invasion and SPI-2 for intracellular proliferation [7, 24]. Two major regulators for the SPI-1 or SPI-2 gene expression are respectively HilA and SsrA.

Several studies describe an increased susceptibility to experimental or natural mucosal infections after the ingestion of some mycotoxins [18, 32, 33]. Hara-Kudo et al. [21] examined the effects of DON on *Salmonella* Enteritidis infection in mice and suggested that administration of 2 mg/L DON in the drinking water reduced resistance to peroral infection of *Salmonella* Enteritidis presumably by inhibiting the cell-mediated immune function. However, until now there are no data available describing a possible interaction between DON and the pathogenesis of a *Salmonella* Typhimurium infection in pigs. The aim of the present study was to examine the effect of DON on the interaction of *Salmonella* Typhimurium with porcine macrophages, at low DON levels that are not cytotoxic and do not impair *Salmonella* growth and virulence gene expression.

2. MATERIALS AND METHODS

2.1. Chemicals

DON, ERK1/2 inhibitor (U0126 ethanolate) [14], ERK1/2 activator phorbol myristate acetate (PMA) [10] and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DON

stock solution of 4 mg/mL was prepared in anhydrous methanol and stored at −20 °C. Serial dilutions of DON were, depending on the experiment, prepared in Luria-Broth (LB, Sigma-Aldrich, Steinheim, Germany) or in the corresponding cell medium allowing the addition of similar volume of vehicle in all experiments. Macrophages seeded onto 24-well or 96-well plates were treated with selected concentrations of DON or equivalent volumes of medium (untreated cells). The ERK1/2 inhibitor U0126 ethanolate was dissolved in DMSO leading to a 3 mM stock solution and stored at −20 °C. For experiments, U0126 ethanolate was diluted in the corresponding cell medium to a final concentration of 10 μM. The PMA stock solution was prepared by dissolving 5 mg of PMA in 1 mL of ethanol and stored in small aliquots at −20 °C. Prior to use, the stock solution was further diluted in the corresponding cell medium to a final concentration of 50 ng/mL.

2.2. Bacterial strains and growth conditions

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a was used as the wild type (WT) strain in which all mutant strains were constructed. To obtain highly invasive late logarithmic cultures for invasion assays [23], 2 μL of a stationary phase culture was inoculated in 5 mL LB and grown for 5 h at 37 °C without aeration. The construction and characterization of a deletion mutant in the gene encoding the SPI-1 translocator/effector protein SipB has been described before [5]. *Salmonella* Typhimurium strains carrying the plasmid containing either the *hilA-luxCDABE* or the *ssrA-luxCDABE* transcriptional fusions were used in the virulence gene expression experiments [5]. For fluorescence microscopy, the pFPV25.1 plasmid expressing green fluorescent protein under the constitutive promoter of *rpsM* was used [6, 35, 36].

2.3. Isolation of porcine pulmonary alveolar macrophages

Porcine pulmonary alveolar macrophages (PAM) were isolated by broncho-alveolar washes from lungs of euthanized 3 to 4 week old piglets, obtained from a *Salmonella*-negative farm, as described previously [12]. The isolated cells were pooled and frozen in liquid nitrogen until further use. Prior to seeding the PAM, frozen aliquots of approximately 10^7 cells/mL, were thawed in phosphate-buffered saline (PBS) with 10% fetal calf serum (FKS, Hyclone, Cramlington, England, UK) at 4 °C. Cells were washed 3 times in PBS and cultured in RPMI (Gibco, Life Technologies, Paisley, Scotland, UK) containing 2 mM L-glutamine (Gibco) and 1 mM sodium pyruvate (Gibco).

2.4. Cytotoxicity assays

The Cell Proliferation Reagent WST-1 kit from Roche Applied Science (Bazel, Switzerland) was used to assess the direct cytotoxic effect of DON on PAM. The test was used according to the manufacturer's instructions. The absorbance of DON-treated wells was measured at 450 nm using a microplate ELISA reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland) and compared with a solvent-treated control. The percentages of DON induced cytotoxicity were calculated using the following formula

$$\% \quad \text{cytotoxicity} = 100 \times \frac{(c-b)-(a-b)}{(c-b)}.$$

In this formula $a = OD_{450}$ derived from the wells incubated with DON, $b = OD_{450}$ derived from blank wells, $c = OD_{450}$ derived from untreated control wells.

In addition to the WST-1 assay, the lactate dehydrogenase cytotoxicity detection kit (LDH, Roche Applied Science) was used to measure cytotoxicity and cell lysis by detecting LDH activity released from damaged cells. The test was used in accordance to the manufacturer's instructions and an ELISA plate reader at 492 nm was used to measure the absorbance.

2.5. Effect of DON on the growth and on SPI-1 and SPI-2 virulence gene expression of *Salmonella* Typhimurium

The effect of concentrations of DON from 0.005 to 20 μg/mL on *Salmonella* Typhimurium WT was examined during 24 h in order to detect a direct toxic effect on the growth of the bacteria. Therefore, *Salmonella* Typhimurium was grown overnight in LB with aeration at 37 °C after which a suspension of the bacteria (Mc Farland 0.5) was added to the different concentrations of DON in a 96-well plate. The plate was incubated at 37 °C and after 24 h of incubation, the number of cfu/mL was determined by titration. This step was carried out by making 10-fold dilutions of 20 μL of the bacterial suspensions. Then six 20 μL samples of each dilution were inoculated on Brilliant Green agar (BGA) and incubated for 24 h at 37 °C after which the colonies were counted.

A FluoroScan Ascent fluorometer (ThermoLabsystems) was used to quantify SPI-1 and SPI-2 expression of *Salmonella* Typhimurium by measuring the light production (luminescence) of *Salmonella* Typhimurium strains carrying the plasmids containing either the *hilA-lux CDABE* or the *ssrA-lux CDABE* transcriptional fusions. Bacterial cultures were grown in microplates in 200 µL of LB medium supplemented with the different concentrations of DON and in non-supplemented LB medium at 37 °C. Light production was measured every 15 min during 24 h and expressed as the relative gene expression.

2.6. Invasion and intracellular survival assays

To examine whether the ability of *Salmonella* Typhimurium and its isogenic *sipB* mutant to invade and proliferate in PAM was altered after preexposure of the macrophages to DON, invasion and intracellular survival assays were performed. Therefore, PAM were seeded in 24-well plates at a density of approximately 5×10^5 cells per well and were allowed to attach for at least 2 h. These wells were exposed to different non-cytotoxic concentrations of DON (0.005–0.100 µg/mL). After 24 h, the wells were rinsed and inoculated with *Salmonella* Typhimurium at a multiplicity of infection (moi) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 *g* for 10 min. After 30 min incubation at 37 °C under 5% CO_2, the wells were rinsed and fresh medium supplemented with 50 µg/mL gentamicin (Gibco) was added for 1 h. For the invasion assay the PAM were lysed with 1% (v/v) Triton-X (Sigma-Aldrich) for 10 min and 10-fold dilutions were plated on BGA plates. To assess intracellular growth, the medium containing 50 µg/mL gentamicin was replaced after 1 h incubation with fresh medium supplemented with 10 µg/mL gentamicin and the number of viable bacteria was assessed 6 h after infection as described above. All measurements were performed in triplicate and the experiment was carried out on three independent occasions.

To visualize the effect of DON on the number of cell-associated *Salmonella* bacteria, PAM were seeded in sterile Lab-tek® chambered coverglasses (VWR, Leuven, Belgium) and exposed to 0.025 µg/mL of DON in cell medium or to cell medium only for 24 h at 37 °C. Subsequently the invasion and proliferation assay was performed as described before after inoculation with green fluorescent protein (gfp)-producing *Salmonella*. Cells were washed three times to remove unbound bacteria. Cell trace® calcein red-orange (Molecular Probes Europe, Leiden, The Netherlands) was added for 30 min at 37 °C. Afterwards, cells were washed three times with PBS and fluorescence microscopy was carried out. In 100 macrophages, the number of macrophages containing gfp-*Salmonella* was counted and the average number of cell-associated bacteria was calculated.

2.7. Morphological changes in macrophages

Staining techniques were performed to visualize possible morphological changes in macrophages exposed to DON in order to explain the observed difference in uptake of *Salmonella* Typhimurium in macrophages whether or not exposed to DON.

2.7.1. Haemacolor staining

PAM were seeded onto 13-mm-circular glass slides (VWR) in a 24-well plate at a concentration of 1×10^6 cells/mL and were incubated at 37 °C in 5% CO_2 for at least 2 h. Subsequently, either 0.025 µg/mL of DON or cell medium was added. After incubation of 24 h at 37 °C in 5% CO_2, cells were washed three times with PBS containing Ca^{2+} and Mg^{2+} (PBS+) and stained with Haemacolor® stain (Merck, Darmstadt, Germany). Glass slides were mounted with coverslips and observed microscopically in at least three time-independent assays. Attention was paid to cellular and nuclear changes with special focus on changes of the cell membrane, presence of membrane ruffles and apoptotic changes in the nucleus.

2.7.2. Staining of F-actin with phalloidin-Texas Red X®

PAM were seeded onto 13-mm-circular glass slides in a 24-well plate at a concentration of 1×10^6 per mL. After 2 h of incubation, the cells were exposed to cell medium, 0.025 µg/mL of DON, 10 µM of the ERK1/2 inhibitor U0126 ethanolate in combination with 0.025 µg/mL DON or 50 ng/mL of the ERK1/2 activator PMA for 24 h at 37 °C in 5% CO_2. PAM exposed to cell medium served as negative control. Thereafter, they were gently washed twice with 0.5 mL PBS+ at 37 °C, fixed with 0.5 mL 3.0% paraformaldehyde for 10 min at room temperature, gently washed again with 0.5 mL PBS+ and then permeabilized with 0.5 mL 0.1% Triton X-100 in PBS+ for 2 min at room temperature. Following washing with PBS+, 0.25 mL phalloidin-Texas Red X® (Molecular Probes, Eugene, Oregon, USA) (1:100 in PBS+) was added to each well and the 24-well plate was

incubated at 37 °C for 1 h. To remove unbound phalloidin, coverslips were gently washed twice with 0.5 mL PBS+ before visualization of the actin filaments with a fluorescence microscope (Leica Microsystem GmbH, Heidelberg, Germany).

2.7.3. Apoptosis and necrosis staining

A caspase-3 staining in combination with ethidium monoazide (EMA; Sigma-Aldrich) and counterstaining with Hoechst was performed to examine whether the observed cell morphology changes were associated with apoptosis or necrosis. Activation of caspases plays a central role in apoptosis with caspase-3 being one of the key effectors. EMA staining was used for the detection of nonviable cells, which can be either apoptotic or necrotic. The Hoechst staining, used as a nucleus staining technique, helped in the determination of cells as apoptotic or necrotic. Macrophages were described as apoptotic when the caspase-3 staining was positive and the EMA staining positive (late apoptosis) or negative (early apoptosis). Necrotic macrophages were negative for caspase-3 but positive for EMA. Normal living cells were negative for both caspase-3 and EMA.

PAM were seeded in a 24-well plate at 1×10^6 per mL and incubated at 37 °C in 5% CO_2 to allow adhesion. After 2 h, 0.025 μg/mL of DON was added for 24 h. Cell medium served as negative control. Macrophages exposed to 3 μM of staurosporine for 3 h served as a positive control for apoptosis. For the staining protocol, cells were detached from the well through incubation with 0.3 mL trypsine for 10 min and pooled in a falcon tube together with the supernatant. After centrifugation (7 min, 365 *g*), the cells were resuspended in 0.5 mL of ice-cold PBS. After another centrifugation step, the supernatant was prelevated and the pellet was resuspended in 0.5 mL of ice-cold EMA (1:20 in PBS). After an incubation step in the dark for 20 min, the falcon tubes were put under a light bulb during 10 min after which they were centrifuged (7 min, 365 *g*). After removal of the supernatant, the cells were fixed by resuspending the pellet in 0.5 mL paraformaldehyde 3% (w/v) in PBS for 10 min at room temperature. After a centrifugation (7 min, 365 *g*) and washing step with 0.5 mL PBS, the cells were permeabilized by incubating in 0.1 mL Triton-X 0.1% for 2 min followed by the addition of 0.9 mL PBS. From this point on, PBS was supplemented with 20% FCS to prevent the cells from sticking to the tube wall. Again, the cells were centrifuged followed by the addition of 0.1 mL of rabbit anti-active caspase-3 (Sigma-Aldrich) diluted 1:100 in PBS. After 1 h incubation at 37 °C, the cells were centrifuged, washed with PBS and afterwards 0.1 mL of goat anti-rabbit FITC (1:50 in PBS) (Sigma-Aldrich) was added. The cells were incubated at 37 °C, protected from light, for 1 h. After centrifugation and washing in 0.5 mL PBS, the cells were counterstained with Hoechst (1 mg/mL, 1/100 dilution in PBS) for 15 min protected from light. After a centrifugation step (7 min, 365 *g*) and a washing step with PBS, the remaining pellet was resuspended in 20–40 μL of PBS and 10 μL was mounted in a small drop of mounting medium (DABCO-glycerine). The cells were observed with a fluorescence microscope.

2.8. Statistical analysis

All experiments were conducted in triplicate with three repeats per experiment, unless otherwise noted. The data were analyzed using ANOVA to address the significance of difference between mean values with significance set at $p < 0.05$.

3. RESULTS

3.1. Low DON concentrations do not affect macrophage viability

The possible direct cytotoxic effect of DON on PAM at concentrations ranging from 0.005 to 50 μg/mL as determined by the WST-1 assay is shown in Figure 1. DON had no significant effect at concentrations $\leq$ 0.100 μg/mL. At 0.250 μg/mL, DON had a significant cytotoxic effect of 85 ± 5% ($p < 0.05$) after 24 h of incubation. Concentrations of DON > 0.250 μg/mL showed a cytotoxicity of more than 97% ($p < 0.05$). The cytotoxicity determined by the LDH test was similar to the previous results (data not shown). Based on these results, only non-cytotoxic concentrations of DON (< 0.250 μg/mL) were used in the further experiments.

3.2. High concentrations of DON increase SPI-1 and SPI-2 expression of *Salmonella* Typhimurium

Concentrations of DON from 0.005 to 50 μg/mL did not have any effect on the growth of *Salmonella* Typhimurium WT (data not shown).

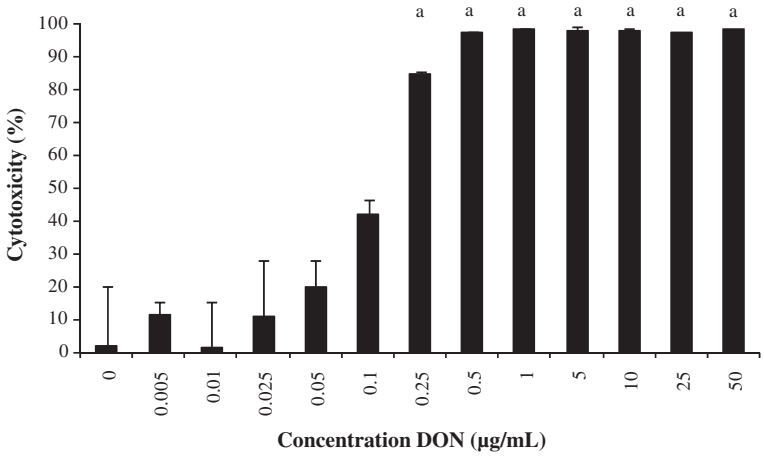

Figure 1. Percentage cytotoxicity in porcine macrophages exposed to DON at 0.005 to 50 µg/mL. Twenty-four hours later, the cytotoxic effect was determined by WST-1 assay. Results represent the means of three independent experiments conducted in triplicate and their standard deviation. Superscript (a) refers to a significantly higher cytotoxic effect compared to the unexposed control wells ($p < 0.05$).

High concentrations of DON ($\geq$ 5 µg/mL) in LB medium significantly increased the expression of *hilA* and *ssrA*, which are regulators required for the expression of respectively the SPI-1 invasion genes or SPI-2 genes necessary for the intracellular replication and systemic infection of *Salmonella* Typhimurium ($p < 0.05$). The results are shown in Figure 2.

3.3. DON promotes the uptake of *Salmonella* Typhimurium in porcine macrophages

The results of the invasion and proliferation test of *Salmonella* Typhimurium WT in PAM with or without prior exposure to DON are summarized in Figure 3A. Uptake was higher in the PAM exposed to 0.025 µg/mL of DON compared to the PAM that were not exposed to DON ($p < 0.05$), with an average increase factor of 1.45. Similar results were obtained using the deletion mutant *sipB* (Fig. 3B). Intracellular replication, represented by log cfu/mL after proliferation minus log cfu/mL after invasion in porcine macrophages, of *Salmonella* Typhimurium WT in porcine macrophages whether or not exposed to 0.025 µg/mL of DON did not differ significantly (0.48 ± 0.339 versus 0.91 ± 0.529 respectively).

The enhanced uptake of *Salmonella* Typhimurium in PAM exposed to 0.025 µg/mL of DON was confirmed in an invasion and proliferation assay with gfp-*Salmonella*. The results of this experiment are summarized in Table I. Macrophages exposed to 0.025 µg/mL of DON were more frequently infected by *Salmonella* Typhimurium in comparison with the control PAM resulting in a higher bacterial count (average 3.9 versus 2.7 bacteria per macrophage, respectively) and a consequent higher number of bacteria in the PAM after 6 h. The proliferation rate of intracellular bacteria however, did not differ significantly between the control and the DON-treated macrophages (2.8 versus 2.6 respectively).

3.4. DON causes morphological alterations and F-actin reorganization in porcine macrophages by activation of ERK1/2

PAM were stained with Haemacolor after exposure to 0.025 µg/mL of DON for an incubation time of 24 h. This staining technique was used to test the hypothesis that DON alters the cell morphology and more specifically induces changes in the cell membranes which might result in an enhanced uptake of *Salmonella*

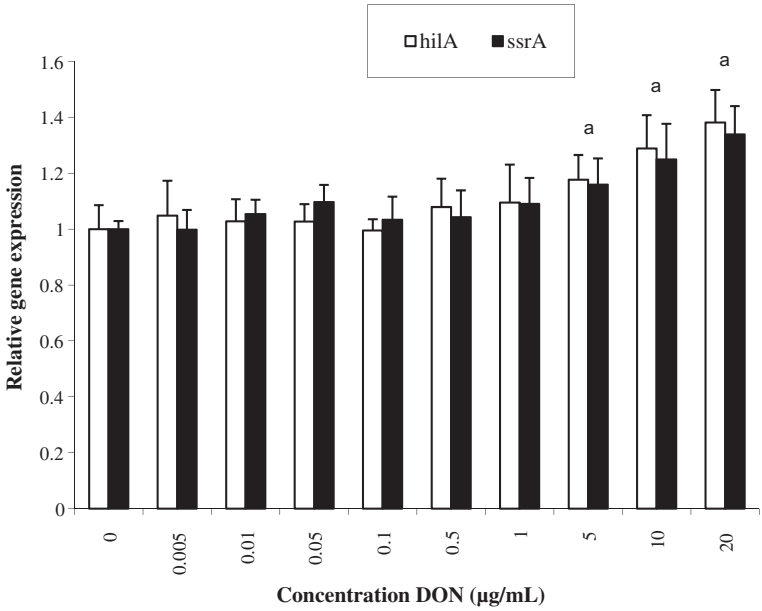

Figure 2. The expression of *hilA* and *ssrA* in *Salmonella* Typhimurium grown in LB medium supplemented with different concentrations of DON. The light production was measured every 15 min during 24 h and is expressed as the relative gene expression. The results represent the means of three independent experiments conducted in triplicate and their standard deviation. Superscript (a) refers to a significantly higher expression of *hilA* and *ssrA* compared to the unexposed control wells ($p < 0.05$).

Typhimurium WT. DON caused marked morphological alterations as the majority of the PAM showed ruffling of the cell (Fig. 4). To examine the actin cytoskeleton, phalloidin was used as a specific probe for polymerized (F) actin. Macrophages exposed to 0.025 µg/mL of DON showed some marked morphological changes compared with the non exposed controls. Macrophages incubated with 0.025 µg/mL of DON for 24 h demonstrated increased formation of ruffling membranes in 78.5 ± 2.26% of the macrophages in comparison with the control macrophages (10.3 ± 3.79%) (Figs. 4C and 4D).

In order to investigate whether the cytoskeletal rearrangements of macrophages after exposure to 0.025 µg/mL of DON, were due to the activation of the MAPK pathway, and more specifically ERK1/2, the actin staining experiment was repeated with on the one hand, DON in combination with the ERK1/2 inhibitor U0126 ethanolate, and on the other hand PMA, which is an ERK1/2 activator.

Macrophages incubated with 10 µM U0126 ethanolate in combination with 0.025 µg/mL of DON, for 24 h, showed a normal F-actin distribution. Only 11.0 ± 2.00% of the macrophages demonstrated ruffling membranes. Macrophages incubated with 50 ng/mL of PMA for 24 h demonstrated increased formation of ruffling membranes in 97.3 ± 2.52% of the macrophages in comparison with the control macrophages (Figs. 4E and 4F) and macrophages treated with U0126 ethanolate (data not shown). These results suggest that the increased membrane ruffling, caused by DON, is due to the activation of ERK1/2.

3.5. DON induced cell morphology changes are not associated with cellular apoptosis and necrosis

To determine the cellular apoptosis and necrosis of PAM exposed to 0.025 µg/mL of DON during 24 h, the EMA, caspase-3 and

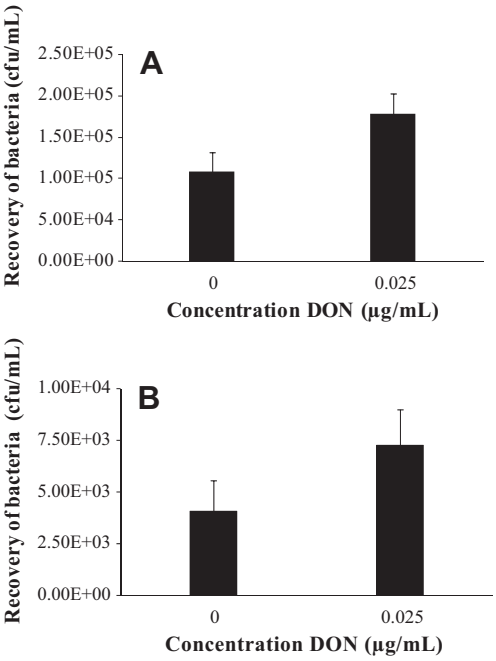

Figure 3. The invasiveness of *Salmonella* Typhimurium WT (A) and its isogenic ΔSipB (B) in porcine pulmonary macrophages whether or not exposed to 0.025 µg/mL of DON is shown. The number of gentamicin-protected bacteria is shown. The results show a representative of three independent experiments conducted in sixfold ± standard deviation for *Salmonella* Typhimurium WT and of two independent experiments conducted in sixfold ± standard deviation for the deletion mutant in *SipB*.

Hoechst staining were examined in 100 macrophages on three independent occasions.

No significant difference was seen between the PAM exposed to 0.025 µg/mL of DON and the control PAM which indicates that this concentration does not induce apoptosis nor necrosis. The results of this experiment are summarized in Table II.

4. DISCUSSION

DON and salmonellosis are emerging issues causing serious problems in the European pig industry. The toxic effects of the mycotoxin DON have been well documented in several animal species and in diverse cell culture experiments. Several publications indicate that the presence of this *Fusarium* mycotoxin in feed can seriously affect the health status of pigs and other animals [16, 28, 30]. *Salmonella* Typhimurium is the predominant serovar isolated from pigs in Europe and since pigs may be subclinically infected, they are an important threat to both animal and human health. Although DON and *Salmonella* can be commonly encountered in the pig industry, this is the first report describing an interaction between DON and the pathogenesis of a *Salmonella* Typhimurium infection in pigs.

Concentrations of DON higher than 0.1 µg/mL had a significant cytotoxic effect on porcine macrophages after 24 h of exposure. These results correspond with the literature where DON was described to inhibit the proliferation of Caco-2 cells in a dose dependent manner, with a significant effect appearing at 0.2 µg/mL [31]. Bimczok et al. [1], however, described that DON only had a significant cytotoxic effect on monocyte-derived dendritic cells at 0.8 µg/mL. To eliminate a possible cytotoxic effect of DON on the porcine macrophages, we chose to work with concentrations of DON below 0.1 µg/mL in the further experiments. These low concentrations are relevant in practice since in several publications, depending

Table I. Results of the invasion and proliferation assay performed with gfp-producing *Salmonella*. The results show a representative of two independent experiments conducted in threefold. The number of cell-associated bacteria was counted in 100 macrophages. The results represent the mean bacterial count per macrophages ± standard error of the mean (sem) after invasion (0 h) and after proliferation (6 h), as well as the proliferation rate.

	Mean bacterial count ± sem		Proliferation rate
PAM	0 h	6 h	
Control	2.7 ± 0.11	7.6 ± 0.35	2.8
0.025 µg/mL DON	3.9 ± 0.13	10.1 ± 0.39	2.6

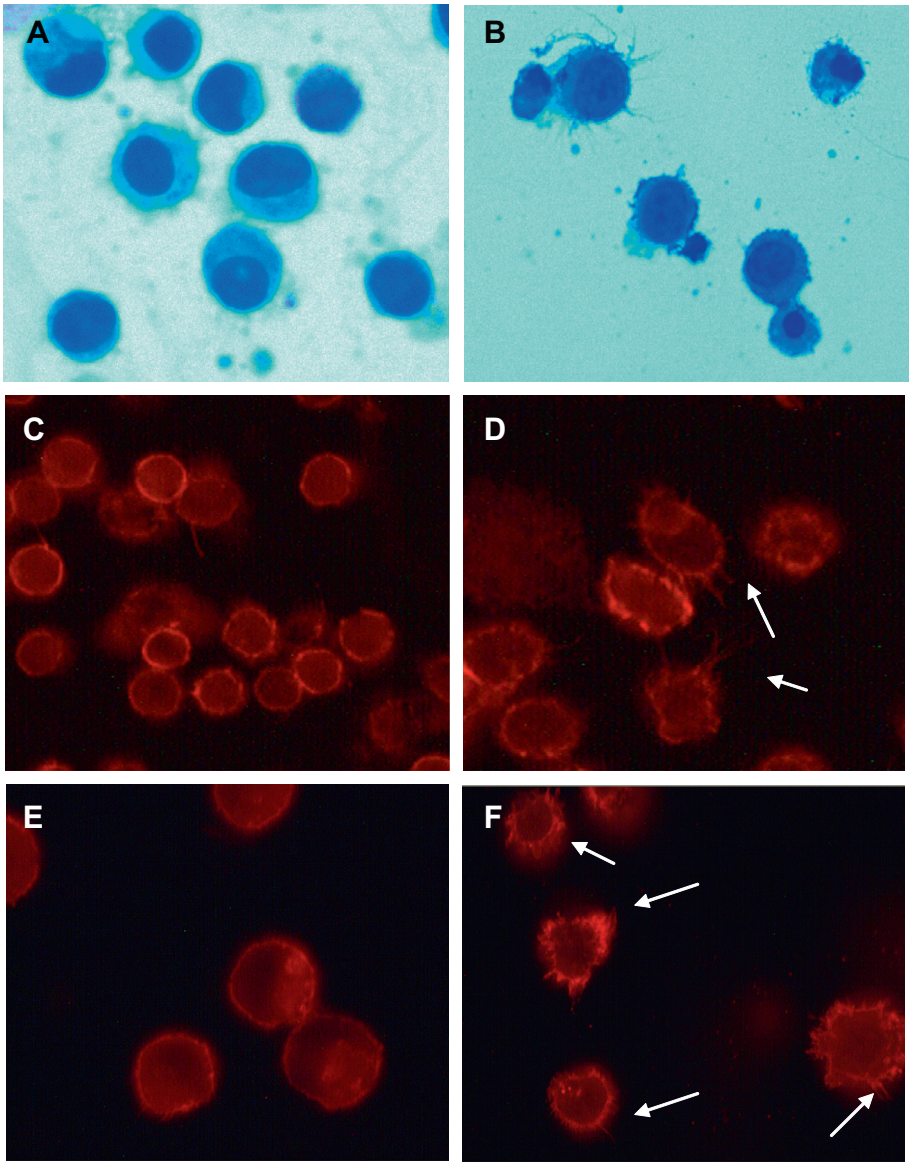

Figure 4. Haemacolor staining of PAM whether or not exposed to 0.025 μg/mL of DON. Figure A shows porcine macrophages not exposed to DON, whereas Figure B shows DON exposed macrophages with marked membrane ruffling. Figures C–F show fluorescence microscopic pictures of actin filament arrangement in PAM either treated with cell medium, 0.025 μg/mL of DON, 10 μM of the ERK1/2 inhibitor U0126 ethanolate in combination with 0.025 μg/mL of DON or with 50 ng/mL of the ERK1/2 activator PMA, for 24 h. Control macrophages (C) and macrophages exposed to U0126 ethanolate in combination with DON (E) demonstrated a normal distribution of F-actin. DON (D) and PMA (F) exposed macrophages demonstrated increased formation of membrane ruffles (indicated by white arrows). (A color version of this figure is available at www.vetres.org.)

Table II. Results of the apoptosis and necrosis staining of PAM whether or not exposed to 0.025 μg/mL of DON. The results represent the average percentage of cells showing apoptosis or necrosis and their standard deviation. Normal cells are negative for both EMA and caspase-3, necrotic cells are positive for EMA but negative for caspase-3, apoptotic cells are always positive for caspase-3 but can be EMA positive (late apoptosis) or negative (early apoptosis).

	Average percentage (%) of cells ± sd		
PAM	Normal	Apoptosis	Necrosis
Control	80.7 ± 6.66	19.3 ± 6.66	0 ± 0
0.025 μg/mL DON	76.3 ± 5.03	21.0 ± 5.00	2.7 ± 2.08

on the dietary content of DON, serum concentrations of DON varying from 0.013 to 0.026 μg/mL were measured in pigs [9, 19].

The growth of *Salmonella* Typhimurium was not affected by concentrations of DON up to 50 μg/mL. Nevertheless, high concentrations of DON (≥ 5 μg/mL) in LB medium, significantly increased the expression of *hilA* and *ssrA*. Although this is an interesting observation, concentrations of 5 μg/mL of DON are exceptional contamination levels under field conditions and hence not relevant for the in vivo situation.

Preexposure of macrophages to concentrations of DON, as low as 0.025 μg/mL during 24 h, enhanced the susceptibility of macrophages for the uptake of *Salmonella* Typhimurium in the macrophages. On average, the colony forming units per mL were 150% higher compared to control macrophages. This was confirmed by microscopic evaluation using gfp-*Salmonella*. Since 0.025 μg/mL of DON was proven not to affect the expression of SPI-1 and SPI-2 genes of *Salmonella* Typhimurium, the observed enhanced uptake might be caused by changes in the cell morphology. This hypothesis is also supported by the results of the invasion test with the ΔSipB strain which gave similar results as the WT strain, indicating that the enhanced uptake seen after exposure of the PAM to DON, is not SPI-1 associated.

Salmonella entry in host cells involves a complex series of actin cytoskeletal changes [20]. When we examined the cell morphology focusing on the cell membrane changes, we saw marked changes in DON-treated porcine macrophages compared to non treated controls. Macrophages exposed to 0.025 μg/mL of DON demonstrated alterations of the cell membrane on Haemacolor staining. Special attention was paid on the ruffling of the cell membrane as this is a cytoskeletal change that is commonly described with the entry of *Salmonella* Typhimurium in the macrophages. Therefore, staining with phalloidin-Texas Red®, as specific probe for polymerized actin, was performed which clearly indicated a reorganization of F-actin and formation of membrane ruffles.

ERK1/2, a MAPK, is activated both by trichothecenes such as DON via a process termed the 'ribotoxic stress response' and by the invasion of *Salmonella* Typhimurium in macrophages [29, 39]. Recently, Yang et al. [37] described that DON induces p21 mRNA stability in human epithelial cells in terms of interactions RNA-binding proteins via ERK 1/2 activation. Since the PAK and the Rho-family of small GTP'ases play a central role in both actin dynamics, membrane ruffling and *Salmonella* induced invasion, p21 mRNA stabilization might explain the changes in the cell membrane morphology seen in macrophages exposed to 0.025 μg/mL of DON and might account for the enhanced uptake of *Salmonella* Typhimurium in porcine macrophages preexposed to DON.

To test the hypothesis that the modulations of the cytoskeleton in macrophages, caused by DON, are due to the activation of ERK1/2, staining with phalloidin-Texas Red® was performed in macrophages after preexposure to an ERK1/2 activator (PMA) or an ERK1/2 inhibitor (U0126 ethanolate) in combination with 0.025 μg/mL of DON.

U0126 ethanolate inhibited the morphological rearrangements in macrophages that were

exposed to DON, whereas PMA caused an increased formation of membrane ruffles. Moreover, PMA and DON-treated macrophages have a similar morphology. These results suggest that the increased membrane ruffling, caused by DON, is due to the activation of ERK1/2.

In conclusion, we have shown that low concentrations of DON could modulate the cytoskeleton of macrophages through ERK1/2 F-actin reorganization resulting in an enhanced uptake of *Salmonella* Typhimurium in porcine macrophages.

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