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1 # *In vivo* effect of deoxynivalenol (DON) naturally contaminated feed on
2 porcine reproductive and respiratory syndrome virus (PRRSV)
3 infection.

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5 Christian Savard[1], Vicente Pinilla[2], Chantale Provost[1], Carl A. Gagnon[1], Younes Chorfi[2].

6 1 Groupe de recherche sur les maladies infectieuses du porc (GREMIP);

7 **Swine and Poultry Infectious Diseases Research Center** (CRIPA), Saint-Hyacinthe,
8 Québec, Canada

9 2 Département de biomédecine vétérinaire, Saint-Hyacinthe, Québec, Canada.

10

11 Corresponding authors

12 Younes.chorfi@umontreal.ca

13 Faculté de médecine vétérinaire, Université de Montréal, 3200 rue Sicotte, Saint-
14 Hyacinthe, Québec, Canada, J2S 7C6

Abstract

Deoxynivalenol (DON), also known as vomitoxin, is the most prevalent type B trichothecene mycotoxin worldwide. Pigs show a great sensitivity to DON, and because of the high proportion of grains in their diets, they are frequently exposed to this mycotoxin. The objective of this study was to determine the impact of DON naturally contaminated feed on porcine reproductive and respiratory syndrome virus (PRRSV) infection, the most important porcine viral pathogens in swine. Experimental infections were performed with 30 animals. Piglets were randomly divided into three groups of 10 animals based on DON content of diets (0, 2.5 and 3.5 mg/Kg DON). All experimental groups were further divided into subgroups of 6 pigs and were inoculated with PRRSV. The remaining pigs (control) were sham-inoculated with PBS. Pigs were daily monitored for temperature, weight and clinical signs for 21 days. Blood samples were collected and tested for PRRSV RNA and for virus specific antibodies. Results of PRRSV infection showed that ingestion of diet highly contaminated with DON greatly increases the effect of PRRSV infection on weight gain, lung lesions and mortality, without increasing significantly viral replication, for which the tendency is rather directed towards a decrease of replication. These results suggest that PRRSV infection could exacerbate anorectic effect of DON, when ingested in large doses. Results also demonstrate a DON negative effect on PRRSV-specific humoral responses. This study demonstrate that high concentrations of DON naturally contaminated feed decreased the immune response against PRRSV and influenced the course of PRRSV infection in pigs.

Keywords: Pig; DON mycotoxin; PRRSV; predisposition to infection

1 **1. Introduction**

2 Various commodities for animal feeding are frequently contaminated with mycotoxins
3 produced by the secondary metabolism of diverse strains of filamentous fungi. Among
4 them, *Fusarium* spp. are the most prevalent mycotoxin producing fungi in temperate
5 regions (Binder et al., 2007). Several toxins are produced by *Fusarium* spp. including
6 trichothecenes deoxynivalenol (DON), nivalenol, and T-2 toxin. *Fusarium spp.* also
7 produces other toxins such as zearalenone (ZEA), fumonisin B1 (FB1), beauvericin and
8 enniatins (Glenn, 2007). Consequently, *Fusarium spp.* naturally infected cereals are
9 frequently contaminated with low levels of several different mycotoxins (Binder et al.,
10 2007).

11 DON, also known as vomitoxin, is the most encountered mycotoxin contaminating cereal
12 worldwide including Canada (Tran et al., 2012). Among farm animals, pigs are the most
13 sensitive animals to DON; dietary concentrations between 2 to 5 mg DON/kg are
14 frequently associated with feed refusal and concentrations over 20 mg DON/kg induce
15 vomiting (Bryden, 2012). DON has a unique effect on immune system as it has the
16 capacity to up and down regulate immune function depending on dose, exposure
17 frequency, timing and the functional immune assay being employed (Pestka, 2008). The
18 molecular mechanism of action of DON imply phosphorylation of mitogen-activated
19 protein kinases (MAPKs) which in turn modulates expression of genes associated with
20 immune response, inflammation and apoptosis. Leukocytes are among the most sensitive
21 cells to DON effect as low concentration of this toxin upregulates immune and
22 inflammatory genes and high concentration triggers cell death, typically by apoptosis,
23 which leads to immunosuppression (Pestka et al., 2004). Immunosuppression engendered

1 by DON has the potential to decrease resistance to infectious diseases (Oswald et al., 2 2005). Previous reports in mice have shown that DON could increase reovirus 3 replication, a double stranded RNA virus, in enteric (Li et al., 2005) and respiratory (Li et 4 al., 2007) infection models. In this latest model, DON exacerbated viral-induced 5 inflammation and pulmonary damage by suppressing type-1 interferon (IFN) response 6 and elevating expression of proinflammatory cytokines (Li et al., 2007). DON has also 7 been shown to modulate the virulence-dependent pathogenesis of infectious bursal 8 disease virus (IBDV) in infected broiler (Danicke et al., 2011). In that study, *Fusarium* 9 contaminated diet, containing predominantly DON at 10.7 mg/kg of diet, significantly 10 increased histopathological lesions of IBDV infected birds. Previous report showed that 11 oral administration of purified FB1 significantly increased the severity of pulmonary 12 lesions following porcine reproductive and respiratory syndrome virus (PRRSV) 13 infection (Ramos et al., 2010). Up to date, no study has reported an interaction of DON 14 with swine viral infections. However, our recent *in vitro* results showed that doses over 15 140 ng/mL of DON could inhibit PRRSV replication (Savard et al., 2014).

16 Swine industry faces many diseases that threaten animal health and the economy of the 17 industry. Among these diseases, PRRS represents the most economically important viral 18 disease of swine industry in North America (Holtkamp et al., 2013). PRRSV causes 19 common clinical signs such as anorexia, fever, and lethargy. In sows, PRRSV is 20 responsible of reproductive failure, characterized by late-term abortions, increased 21 numbers of stillborn fetuses, and/or premature, weak pigs. Furthermore, PRRSV is 22 responsible of respiratory problems in growing and finishing pigs. Respiratory problems 23 induce by PRRSV are usually more severe in young piglets and often aggravated by co-

infections with bacterial and viral pathogens (Chand et al., 2012; Dorr et al., 2007). PRRSV is an enveloped, single stranded, positive sense RNA virus belonging to the *Arteriviridae* viral family, which includes lactate dehydrogenase-elevating virus (LDV) of mice, simian hemorrhagic fever virus (SHFV) and equine arteritis virus (EAV) (Meulenberg et al., 1994).

Pigs are frequently exposed to DON because of their cereal-rich diet that is frequently contaminated by *Fusarium spp.* mycotoxins. Chronic exposure of pigs to DON could impair immunity and decrease resistance to infectious diseases, on the other hand *in vitro* exposure to DON could inhibit PRRSV replication. Therefore the objective of this study was to evaluate the impact of DON naturally contaminated feed on PRRSV *in vivo* infection.

2. Materials and Methods.

2.1. Animals

The experiment was conducted at the Faculté de médecine vétérinaire, Université de Montréal. Animal care procedures followed the guidelines of the Canadian Council on Animal Care and the protocol was approved by the Institutional Animal Care Committee (Protocol #11-Rech-1609). Thirty commercial crossbred piglets, negative for PRRSV were purchased locally at 4 weeks of age. After one week of acclimation on a commercial ration, piglets were randomly divided into 3 experimental groups of 10 animals, housed separately and fed naturally contaminated diets containing 0, 2.5 or 3.5 mg/kg of DON for all the duration of the experiment.

2.2. Experimental diets

Experimental diets (Table 1) were formulated according to the energy and amino acid requirements for piglets as previously described in the National Swine Nutrition Guide (2010). Wheat used in experimental diets was naturally contaminated with DON. Dietary contents of mycotoxins (Table 2) were analysed in the final diet through ultra-performance liquid chromatography/electrospray ionization tandem mass spectrometry, based on method of (Jackson et al., 2012).

2.3. PRRSV challenge strain and experimental infection

PRRSV isolate used in this study was FMV12-1425619 (Genbank accession number KJ888950), obtained from one serum sample originating from a PRRS clinical case. Based on ORF5 phylogenic analyses, this strain, often associated with clinical signs reported from the field, was classified within a lineage 1 of type II genotype cluster that is frequently found in Quebec over the past 2 years (data not shown). Since several different

attempts to isolate the virus have failed, the viral inoculum was obtained from a lung tissue homogenate after infection of piglet with 3 mL of PRRSV positive serum, 2 mL intranasally (i.n) and 1 mL intramuscularly (i.m), containing 1.7 X 10^3 $TCID_{50}$/mL PRRSV. PRRSV concentration in filtrated lung tissue homogenate was determined by RT-qPCR to be 1.5 X 10^4 $TCID_{50}$/mL. It was also determined by PCR that the tissue homogenate was negative for bacteria (with a 16S gene amplification by PCR) (Cai et al., 2003), swine influenza virus (Tremblay et al., 2011), porcine parvovirus (Gagnon et al., 2007) and porcine circovirus (Gagnon et al., 2008). A pilot study using four animals confirmed the capacity of the virus containing inoculum to induce PRRSV-specific clinical signs, viremia, and lung lesions (data not shown).

After 2 weeks on the experimental diets, all experimental groups were further subdivided in groups of 6 pigs, kept in separated rooms and inoculated i.m with 1mL containing $1.5x10^4$ $TCID_{50}$ PRRSV and i.n with 1mL of the same inoculums in each nostril. Remaining pigs of each experimental groups (4) were housed separately and were sham-inoculated with PBS buffer.

2.4. Body weight, rectal temperature and blood collection.

Pigs were daily monitored for rectal temperature, body weight and clinical signs for 21 days post-infection (p.i). The average daily gain (ADG) was calculated by subtracting the initial body weight of the final body weight and divided by the number of experimental days. Fever was defined as body temperature higher than 40˚C for two consecutive days.

Blood samples were collected at days -1, 3, 6, 9, 14 and 21 p.i and PRRSV viremia was evaluated by RT-qPCR and serological response by ELISA. Serum samples were stored frozen for further analysis.

2.5. Macroscopic and microscopic lung lesions evaluation.

Pigs were euthanized on day 21 p.i, and macroscopic lung lesion scores were recorded to estimate the percentage of lung affected by pneumonia (scores vary from 0 to 100%). Each lung lobe was assigned a percentage to reflect the approximate volume of the lobe on the entire lung, based on lung schematisation of (Sorensen et al., 2006). Lung samples and tracheobronchial lymph nodes were fixed in 10% neutral buffered formalin to evaluate specific microscopic lesions. Lung samples were collected to evaluate viral load and stored frozen until tested. Lung sections were scored for severity of interstitial pneumonia as follows 0= normal, 1= mild, 2=moderate, 3= severe, 4= severe with alveolar disappearance. Presence of leucocytes, serum, or necrotic debris in alveolar exsudate were also scored as follows 0=normal, 0.5 rare, 1= mild, 2= moderate, 3= important, and 4= severe. Finally lymphoid follicular hyperplasia were scored as follows 0=normal, 1=mild, 2=moderate, 3= severe.

2.6. PRRSV quantification

Sera and lung homogenates were analyzed for the presence of PRRSV RNA using RT-qPCR assay as described by (Gagnon et al., 2008). Lung tissues were weighed, an equal volume of PBS added to sample and tissues were homogenized before viral RNA isolation. QIAamp Viral RNA kit (Qiagen) was used to isolate viral RNA from serum samples and lung homogenates according to the manufacturer's instructions. A commercial PRRSV RT-qPCR diagnostic kit (NextGen, Tetracore Inc., Gaithersburg, MD, USA) was used for PRRSV quantification as recommended by the manufacturer. The quantification of PRRSV was determined by comparing the sample results with a standard curve based on the amount of serially diluted PRRSV IAF-Klop reference strain

produced in MARC-145 cells and titrated as $TCID_{50}$/mL in the MARC-145-infected cell (Gagnon et al., 2008). The PRRSV RT-qPCR results were expressed in $TCID_{50}$/mL of serum or g of lung tissues.

2.7. PRRSV specific antibodies

Sera were assayed for virus-specific antibody by ELISA with the Herdchek PRRS X3 diagnostic kits (IDEXX Laboratories, Portland, Maine, USA). Serum were diluted 1/40 in diluents supplied by the manufacturer and the assay was performed following the manufacturers' instructions. A sample-to-positive (S:P) ratio equal or greater than 0.4 was considered positive.

2.8. Statistical analysis

Results are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism software (version 5.03, GraphPad Prism software Inc., San Diego, CA). Data were statistically analysed using a one-way ANOVA with Dunnett's multiple comparison test, using animal receiving control diet as control group. For the ADG data, noninfected versus infected animals were compared by applying Student′s unpaired ′t′ test, for each DON concentration. For viremia, lung viral load and microscopic lesions, pair-wise mean comparisons between control and DON treated animals were made using Student′s unpaired ′t′ test. $P<0.05$ was considered to reflect statistically significant differences.

3. Results

3.1. Growth performance

Results of growth performance showed that noninfected pigs had significantly higher ADG than PRRSV infected pigs (Fig. 1A) regardless of DON contamination of the diets. Severe growth retardation was observed following PRRSV infection suggesting a high degree of virulence associated to FMV12-1425619 strain used for the experimental infection. Contaminated diet with DON at 3.5 mg/kg decreased significantly ($P<0.05$) the ADG of noninfected group with a loss of approximately 19% of kg/day compared to uncontaminated noninfected group (Fig. 1A). Same level of contamination in infected animals had severe impact since they had a 40% of ADG less than PRRSV infected animals fed control diet ($P<0.05$) (Fig. 1A). These data suggest that pigs fed high mycotoxin diet are more affected by PRRSV than those fed uncontaminated diet, and the anorectic effect of DON could be additive to that of PRRSV infection.

One PRRSV infected pig in control diet group and one PRRSV infected pig in 2.5-mg/kg group were humanely sacrificed at day 16 and 17 p.i, respectively. At necropsy, lungs of both animals showed PRRSV associated lesions covering over 30% of the organ. At microscopic level, lungs section of both animals showed interstitial pneumonia with presence of leucocytes and necrotic debris in alveolar exudate. Two PRRSV infected pigs in 3.5-mg/kg group were found dead at day 12 and 20 p.i. Due to damage caused by post mortem freezing, the accurate assessment of PRRSV specific lesions has been made difficult. All data from these pigs were excluded from further analysis. Mortality rate due to infection was relatively high, being above 15% for all infected groups (Fig. 1B). Mortality in the PRRSV infected group ingesting 3.5 mg/kg of DON contaminated diet

was at 33% but not statically significant, due to the small number of pigs included in the study. All noninfected pigs survived the experiment (data not shown).

3.2. Body temperature, viremia and viral loads in the lungs.

Five out of 6 PRRSV infected pigs fed uncontaminated diet and 4 out of 6 fed 2.5 mg/kg diet had fever over 6 days (Fig. 2A). In contrast, no pig from PRRSV infected group receiving diet contaminated at 3.5 mg/kg of DON experienced fever episode. Temperature of all noninfected animals remains normal for all duration of the study (Fig. 2B).

All experimentally infected pigs, regardless of DON contamination levels of the diet, developed viremia from day 3 p.i to the end of the experiment (Fig. 3A). Viremia was significantly higher in DON contaminated groups early after experimental infection at day 3 p.i for 2.5 mg/kg DON treated group and at day 6 p.i for 3.5 mg/kg DON treated group suggesting an acceleration of viral replication kinetics in presence of DON (Fig. 3A). However, DON had a limited impact on viremia after day 9 p.i since viremia was similar between all experimental groups (Fig. 3A). Evaluated at day 21 p.i, viral load in the lungs was also increased in all experimentally infected animals (Fig. 3B) compared to noninfected animals that were negative (data not shown). DON contamination appeared to be associated to PRRSV elimination in the lungs, since viral loads of this organ were respectively 5 and 6 times lower in pigs fed diet 2.5 mg/kg (P=0.0965) and 3.5 mg/kg (P=0.0195) of DON than pigs receiving uncontaminated diet (Fig. 3B).

3.3. Macroscopic and microscopic lesions

Following necropsy, observations were made of the macroscopic lung lesions of each piglet. Extents of lesions were recorded as percentage of lungs affected by pneumonia.

Macroscopic lesions were observed on most of the lungs of animals that survived the infection. The extent of lung lesions was significantly more important in animals fed 3.5 mg/kg DON diets (Fig. 4A) than the other groups. No significant PRRSV-associated lung lesions were observed in noninfected animals (data not shown). Microscopically, lungs of all infected pigs showed a severe interstitial pneumonia. However, infected group fed 3.5 mg/kg DON had significantly higher presence of leucocytes, serum, and or necrotic debris in alveolar exsudate (Fig. 4B). Analysis of lymph nodes did not reveal significant changes (data not shown).

3.4. Antibody response

As demonstrated by measured specific antibodies against PRRSV in the serum, all experimentally infected animals, regardless of DON contamination, had seroconverted (Fig. 5). In animals fed uncontaminated diet, humoral response was significantly lower ($P<0.05$) in animals ingesting 2.5 mg/kg of DON but was similar to that of animals fed 3.5 mg/kg (Fig. 5). Noninfected animals did not PRRSV seroconverted.

4. Discussion.

Mycotoxins are frequently detected in different sources of grains designated to animal feeding. Among these mycotoxins, DON is the one that draws the most attention because of its frequent occurrence at levels high enough to cause adverse effects, particularly in pigs. The main clinical effect of DON at lower dietary concentrations is anorexia and decreased weight gain (Danicke et al., 2004; Goyarts et al., 2005; Rotter et al., 1994). The reduced weight gain observed following ingestion of our experimental diets is consistent with what has been previously observed.

In this study, growing young piglets have been used as a model for experimental infection with PRRSV that can cause many different clinical manifestations including fever, lethargy and severe pneumonia often complicated by concurrent bacterial infections (Done et al., 1996; Rossow, 1998). But this clinical picture is highly variable, ranging from mild subclinical infection to acute death of infected animals (Mengeling and Lager, 2000). Several factors can influence the severity of PRRSV infection including host genetics, management practices, environmental factors, concurrent bacterial infections and virus strain heterogeneity (Goldberg et al., 2000; Halbur et al., 1995; Halbur et al., 1996). Environmental factor such as the presence of DON in animals feed could impact on the severity of PRRS disease following an experimental infection. Like DON, PRRSV has been shown to cause anorexia and reduction of weight gain (Done et al., 1996; Rossow, 1998). Results of experimental infections showed that ingestion of diets highly contaminated with DON greatly increases the effect of PRRSV infection on reduction of weight gain. Both effect appears to be additive.

Nonetheless, animals in this study were fed naturally contaminated diets. It is well known that *Fusarium* naturally infected cereals are commonly contaminated with low levels of several different mycotoxins (Binder et al., 2007). In this study, FB1 was the second most abundant mycotoxin found after DON. In swine, FB1 can have negative impact on production by causing pulmonary edema, liver failure or cardiovascular toxicity (Haschek et al., 2001). FB1 has also been shown to increase the severity of pulmonary lesions following porcine reproductive and respiratory syndrome virus (PRRSV) infection (Ramos et al., 2010). However, the FB1 concentrations found in our experimental diets are well below levels that may cause clinical signs according to Ramos et al. (2010). However, we cannot exclude that they could, in part, be responsible for the observed effect. Co-occurrence of FB1 and DON in animal feedstuffs is common and it has been shown that subclinical co-exposure of pigs to these toxins resulted in greater immune suppression than exposure of a single toxin (Grenier and Oswald, 2011). A significant amount of DON was also found in a conjugated form of DON, the DON-3-glucoside. It is not surprising to found this form of conjugate because it was estimated that this conjugate could constitute up to 20% of the total content of its mycotoxin precursor (Berthiller et al., 2006). Hydrolysis of this conjugate, following ingestion, may thus increase exposure to the precursor toxin.

In addition to anorexia, DON has also demonstrated a unique ability to up- and down-regulate immune functions (Pestka et al., 2004). As consequence, DON has been shown to exacerbate some viral infection in mice (Li et al., 2007; Li et al., 2005) and in broiler chicken (Danicke et al., 2011). Here, results of the study showed an increase of viremia during the first days' post-infection following chronic ingestion of DON. This suggests

1 that DON could accelerate the kinetics of viral replication by an unknown mechanism.
2 This result is in contradiction with our previous *in vitro* study showing that DON, at
3 concentration over 140 ng/mL could inhibit PRRSV replication (Savard et al., 2014).
4 This could be explained by the fact that *in vivo* exposure to DON in this study was not the
5 same under *in vitro* conditions. Indeed, it was previously shown that the maximum blood
6 level of DON was approximately 20 ng/mL following ingestion of contaminated diet at
7 4.5 mg / kg of DON (Goyarts and Danicke, 2006), this level similar to the highest
8 contamination doses used in the present study, which is probably insufficient to have the
9 DON inhibitory effect. However, the viremia was not modulated by DON, at the end of
10 the viremic period, between days 9 and 21 p.i. Viral load in the lungs, observed at 21
11 days p.i was significantly decreased by chronic consumption of DON. This suggests that
12 virus elimination could be accelerated in the presence of DON. This is in contradiction
13 with previous reports which reported an increased lung and intestinal reovirus burden and
14 suppression of viral clearance in mice (Li et al., 2007). Unlike its effect on blood viremia,
15 DON is more inhibitor on viral load in the lungs, which is in agreement with our previous
16 study. PRRSV is responsible of specific lung lesions that vary from no apparent to severe
17 tan consolidation lesions that are frequently aggravated by lesions resulting from
18 concurrent bacterial infection (Rossow, 1998). Here, PRRSV specific macroscopic lung
19 lesions were significantly increased by the chronic absorption of DON, when present in
20 larger quantity. Since lung viral load appears to be lower in DON exposed animals, the
21 increased clinical effects in these groups could not be explain by increased viral
22 replication. To some extent, the increased clinical and pathological effects of PRRSV can
23 be caused by enhanced inflammatory immune response rather than the higher levels of

virus replication (Morgan et al., 2013; van Reeth and Nauwynck, 2000). Since DON has been previously shown to stimulate the expression of pro-inflammatory cytokines in lungs of mice (Amuzie et al., 2008), excess production of pro-inflammatory cytokines following DON exposure can possibly explain the higher clinical and pathological effects seen in PRRSV infected animal fed high DON diet. Further investigations will be needed to confirm this hypothesis. Microscopically, PRRSV-specific lung lesions were characterized by septal tickening and presence of alveolar necrotic debris, macrophages and other mononuclear cells. Higher level of DON contamination significantly increases the presence of alveolar necrotic debris, macrophages and other mononuclear cells. These results are consistent with the previous hypothesis.

PRRSV infections normally induce an abundant virus-specific antibody response with minimal virus neutralization activity (Kimman et al., 2009). Our results showed that dietary DON at 2.5 mg/kg significantly decreases PRRSV specific humoral responses. PRRSV vaccines are important tools in the effort to control the disease. Up to date many PRRSV vaccines have been developed, including products that contain modified live virus (MLV) derived from cell culture attenuation of virulent field isolates (e.g. Ingelvac® PRRS MLV and Porcilis® PRRS) (Murtaugh and Genzow, 2011). Presently, only MLV-PRRSV vaccines offer good protection towards homologous strains and mitigated efficiency against anti-genically distant heterologous isolates (Murtaugh and Genzow, 2011; Zuckermann et al., 2007). Here we showed some indication that immune response against virulent strain could be affected in presence of mycotoxins and that could potentially affect the efficiency of MLV vaccines.

In conclusion, results of this study reveals that anorectic effect of DON is additive to anorectic effect of PRRSV, aggravating clinical signs of the infection, when ingested in higher concentration. At level frequently encounter in the field, anorectic effect of DON had more important impact than its inhibitor effect on PRRSV replication.

Acknowledgements

The authors gratefully thank the Canadian Swine Research and Development Cluster for funding this project. C Savard and C Provost received postdoctoral fellowships from the Canadian Swine Health Board (CSHB) and C Savard from the Fonds de recherche du Québec - Nature et technologies (FRQNT). V Pinilla received a scholarship from Innovagrains Network. The authors would also like to thank Dr Sonia Chénier for her assistance in histopathology analysis. PRRSV stain production was supported by Zoetis Canada.

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34

1 Table 1. Diet compositions.

Ingredient g/kg diet	Control diet (0 mg/kg DON)	Diet #1 (2.5 mg/kg DON)	Diet # 2 (3.5 mg/kg DON)
Wheat (0 mg/kg DON)	713.35	543.35	389
Wheat (9 mg/kg DON)	0	167	324.5
Soybean meal	100	100	100
Lactoserum	75	75	75
Fat	15	15	15
Phosphate Dical.-21%	12	12	12
Limestone	12	15	12
Salt	3	3	3
Threonine	3	3	3
Methionine	1.65	1.65	1.65
Se mg/kg	0.4	0.4	0.4
Vitamin E 10%	0.25	0.25	0.25
Cu chloride 58%	0.15	0.15	0.15

2

3 All diets were formulated to reach the following requirement: metabolisable energy 3200 kcal/kg,
4 protein 19%, fat 3%, fiber 2.5%, moisture 10%, Ca 0.8%, Mg 509 mg/Kg, total P 0.7%, K 0.7%,
5 Na 0.2%, Se 0.6 mg/Kg, Cu 120.4 mg/kg, Zn 250 mg/Kg, vitamin A 18.4 KIU/Kg, vitamin D 2.5
6 KIU/Kg, vitamin E 106.7 mg/Kg, biotin, 0.3 mg/Kg.

7

1 Table 2. Mycotoxins' content of the diets.

	Diets		
Mycotoxin contamination (mg/kg)[a]	0	2.5	3.5
Ochratoxin A	0.0043	N.D[b]	N.D
Ochratoxin B	0.0028	N.D	N.D
Deoxynivalenol	0.2782	2.6680	3.7553
3-AcDon	N.D	0.0227	0.0220
15-AcDon	N.D	N.D	0.0340
DON-3-Glucoside	0.0134	0.1750	0.2732
Fumonisin B1	0.4440	0.4596	0.4385
Zearalenone	N.D	0.2342	0.2091
Mycophenolic Acid	N.D	0.0033[c]	0.0021[c]
Wortmannin	N.D	0.0011[c]	N.D
Verruculogen	N.D	N.D	0.0100
Ergometrine/Ergonovine	0.0045	0.0020	0.0013[c]

2 [a]Value of detected mycotoxin in at least one diet only.

3 [b]N.D: Not detected, values below limit of detection.

4 [c]Values under the limit of quantitation but above limit of detection.

Figure

1 Figure1. Effect of DON naturally contaminated diets on piglet's growth following PRRSV
2 infection.

3 Groups of piglets (10) were fed with DON naturally contaminated diets (2.5 and 3.5 mg/kg of
4 feed) for 2 weeks. A control group of piglets received uncontaminated diet for the same period of
5 time. A subgroup of piglets (n=6) was PRRSV infected and the remaining piglets (4) were sham
6 infected with PBS. Average daily gain was calculated by dividing the total weight gain on the
7 number of days of the study (A). Kaplan-Meier survival curve in PRRSV infected animals with
8 end points piglet death (B). $^{+++}$significant when compared to respective PRRSV infected group
9 ($P<0.001$).
10 *significant when compared to control group (0 mg DON/kg) ($P<0.05$).

11 Figure2. Time course of body temperature (°C) during experimental infection.

12 PRRSV infected (A) and noninfected (B) pigs were monitored daily for rectal body temperature
13 during 21 days post-infection. Fever was defined as rectal temperature higher than 40˚C for two
14 consecutive days.

15 Figure3. Effect of DON naturally contaminated diets on PRRSV viremia and lungs viral load.

16 Blood was collected at day -1,3, 6, 9, 13, and 21 pi and serum tested for the presence of PRRSV
17 RNA by RT-qPCR (A). At necropsy, sections of lung were collected to determine the pulmonary
18 viral load by RT-qPCR (B). Data are expressed in $TCID_{50}$/mL. *significant when compared to
19 control group (0 mg DON/kg) at the same day ($P<0.05$). Note: animals sacrificed during the
20 experiment were excluded from the analysis.

21 Figure 4. Effect of DON naturally contaminated diets on macroscopic and microscopic lung
22 lesions in PRRSV infected animals.

At necropsy, on day 21 p.i, macroscopic lung lesions were recorded as percentage of lungs affected by pneumonia (A) and microscopic presence of leucocytes, serum, or necrotic debris in alveolar exsudate were scored (B). *significant when compared to control group (0 mg DON/kg) at the same day (*P*<0.05). Note: animals sacrificed during the experiment were excluded from the analysis.

<u>Figure 5. Effect of DON naturally contaminated diets on PRRSV specific antibody response.</u>

Blood was collected at day -1, 9, 13, and 21 p.i and sera were tested for the presence of specific PRRSV antibodies using a commercial ELISA kit (HerdChek-PRRS®,IDEXX). Data are expressed in sample to positive ratio. *significant when compared to control group (0 mg DON/kg) at the same day (*P*<0.05). #tendency when compared to control group (0 mg DON/kg) at day 21 (*P*=0.09)

1 Figure 1.

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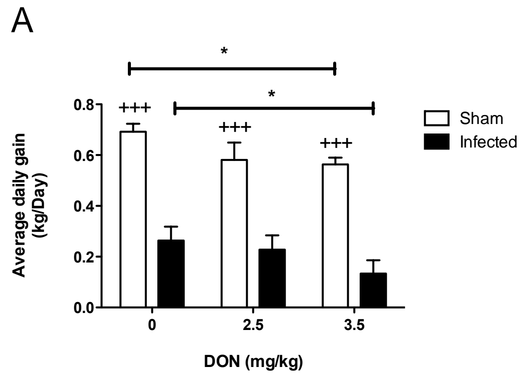

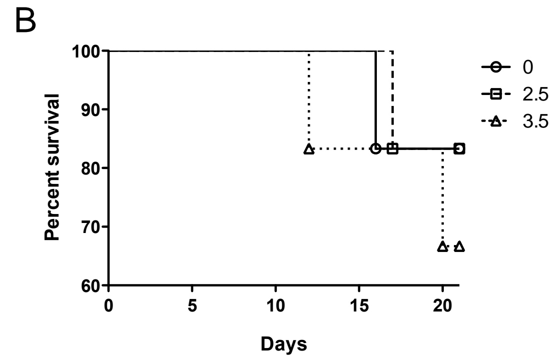

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1 Figure 2.

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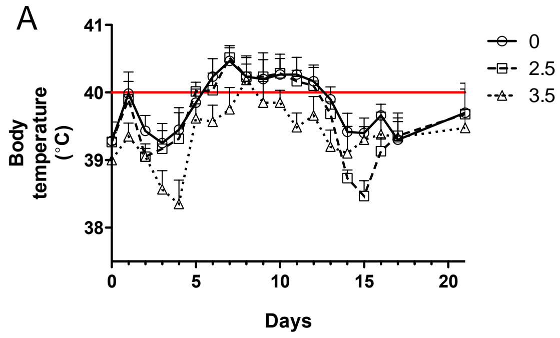

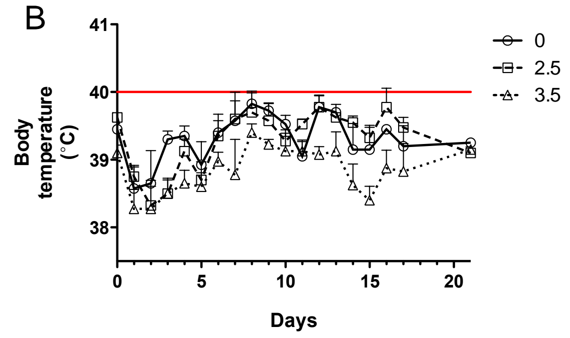

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1 Figure 3.

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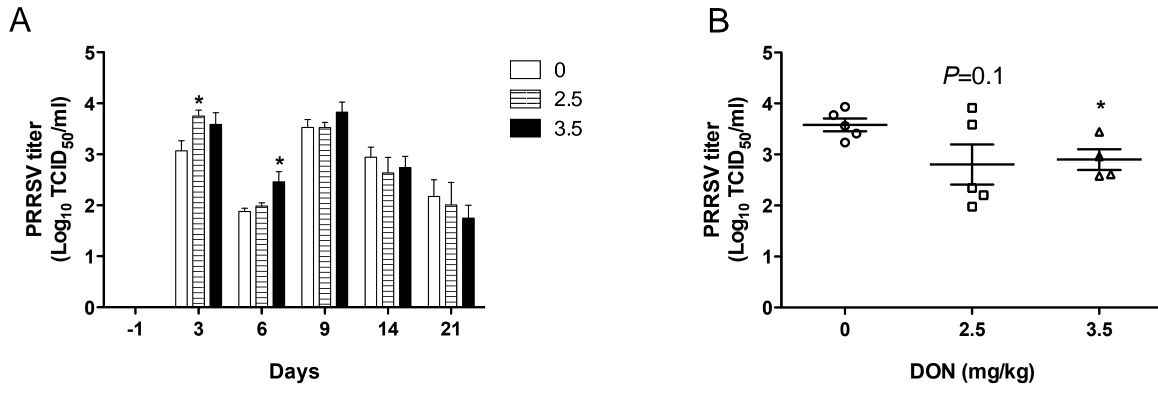

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1 Figure 4

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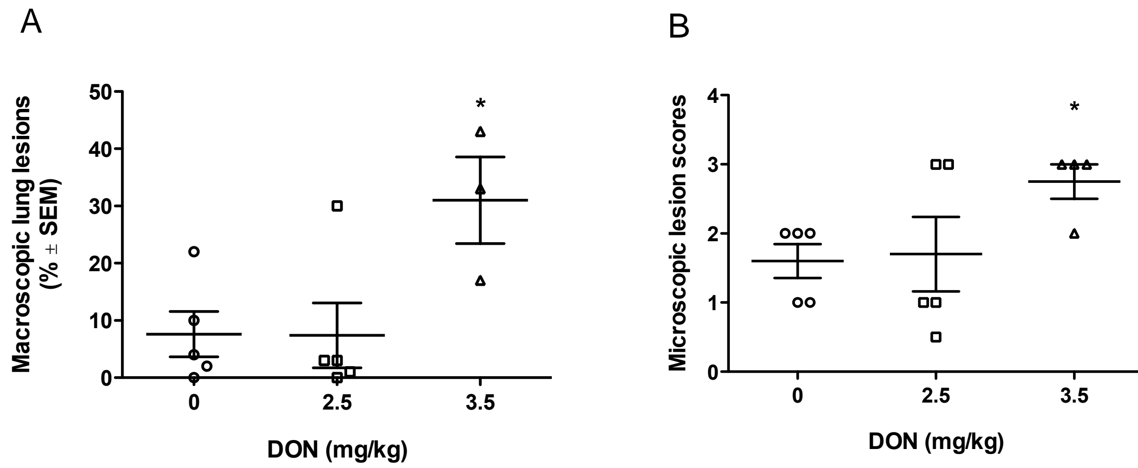

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1 Figure 5

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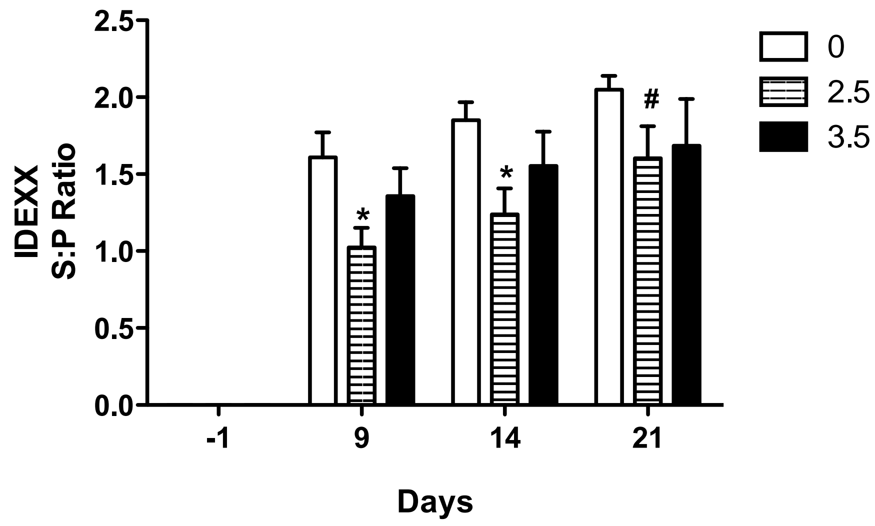

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