Canadian Swine Research and Development Cluster (CSRDC)
Grappe porcine canadienne de recherche et de développement (GPCRD)

FINAL PROJECT REPORT

April 2010 - December 2012

Project Title

Efficacy of feed additives to mitigate the negative impacts of mycotoxin contaminated feed on performance and health of piglets

CSRDC project #

1014

Prepared by

Frédéric Guay

Organization name

Université Laval

Date

February 2013

Executive Summary

Briefly summarize the activities funded in this project, highlighting key achievements and results. Also, include a few sentences to answer this question: Why are the project and results significant for the target group and/or stakeholders? Explain any significant discrepancy between the achieved results and the planned results.

Activity 2.

Objective 1a. The analysis of cell viability and mortality presented in this study showed that concentration of 560 ng/ml and higher were significantly detrimental to the survival of MARC-145 and PAM cells. Cell viability and mortality showed a rapid cell death caused by PRRSV strains used in this study. Interestingly, exposure to sub-toxic DON diminished significantly cell mortality triggered by PRRSV, in a dose dependant manner. Our data also showed that specific PRRSV antigen content of infected cells was diminished as seen in the immunofluorescence experiment. This result was confirmed by qPCR indicating a lower amount of PRRSV genes after DON exposure. These results suggest that sub-toxic DON doses could inhibit PRRSV replication which may explain decreased cell mortality caused by DON in infected cells.

Objective 1b. This study demonstrated that NPTr cells are also very sensitive to the toxic effect of DON and that this effect is more pronounced at the cell viability level than the cell mortality level. In addition, the effect of DON on PCV2 infected NPTR cells was dose and genotype dependant. In fact, all the results of this study suggest that replication of PCV2b could be enhanced by the addition of DON, at sub-toxic concentrations while those of PCV2a would be rather reduced. However, high concentration of DON seems to strongly reduce viral replication of PCV2 regardless of genotypes, potentially by affecting cell survival. Interferons mRNA expression analysis reveal that IFN-β mRNA expression is inhibited in PCV2a infected cells, while it is increased in PCV2b infected cells, which could partly explain the difference in replication between the two genotypes.

Objective 2a. The main results of the study showed that ingestion of diets highly contaminated with DON greatly increases the effect of PRRSV infection on weight loss, lung injury and mortality, without increasing significantly viral replication, for which the tendency is rather directed towards a decrease of replication. Taken all together, these results suggest that PRRSV infection could exacerbate the anorectic effect of DON, when ingested in large doses. Like the ration of 3.5 ppm / kg, the ration at 1.5 ppm/kg reduces PRRSV replication, however these diets seem rather to decrease lesions in the lung, but have no significant impact on clinical signs. These results also demonstrate a negative impact on antibody response of pigs fed DON contaminated diet. DON could therefore undermine the efficacy of live attenuated vaccine against PRRSV by interfering with the humoral response of animals.

Objective 2b. In the light of our results, no clear potentiating effect of DON mycotoxin was observed under the present experimental conditions in development of PCV2 associated diseases, even if the replication of the virus was slightly increased in the group ingesting feed containing 1.5 ppm/kg of DON. However, results on growth performances tend to demonstrate a beneficial effect of DON rather than the development of clinical signs. Further experiments will be necessary to confirm this tendency, probably by using more virulent strains, or adding one another triggering agent such as PRRSV. These results also demonstrate a negative effect on antibody response of pigs fed a diet highly contaminated with DON. High concentration of DON could therefore undermine the efficacy of a live attenuated vaccine directed against PCV2 by interfering with the humoral response of the animal.

Activity 3. For this experiment, sixty piglets were used to evaluate the effect of DON (deoxynivalenol) and four dietary supplements on growth performance and nutrient digestibility of weanling pigs. We used the following six treatments: treatment 1 (positive control, feed not contaminated with DON, <0.5 ppm), treatment 2 (negative control, DON-contaminated feed, 4 ppm), treatment 3 (negative control + Integral), treatment 4 (negative control + Biofix), treatment 5 (negative control + MXM), and treatment 6 (negative control + Defusion). Weaned piglets (21 days old) were housed individually and randomly received one of the six treatments for a period

of 14 days after weaning. Growth and feeding efficiency were determined during this period, and a digestibility test performed during the last 5 days. Blood samples were taken to determine serum concentration of DON. So far, the results indicate that DON-contaminated feed decreases growth performance while Defusion supplement restores performance in piglets fed with contaminated feed.

The results showed that there are differences in the effectiveness of various feed supplements (mycotoxin inhibitor products) to counteract the negative effect of mycotoxin contaminated feeds on growth performance. In addition, the four mycotoxin inhibitor products evaluated show varying effects on the digestibility of nutrients (Ca, P, N, energy), particularly with respect to nitrogen. If these differences are corroborated in field trials, it would mean that judicious selection of commercial mycotoxin inhibitors could have a considerable impact on the profitability of the swine producer.

An additional benefit derived from this project will be the availability of a serological test for early detection of DON contamination in piglets, thus providing a means to monitor contamination and/or the effectiveness of feed supplements to counteract such contamination.

Comparison of the four commercial mycotoxin inhibitors (Integral, Biofix, MXM and Defusion) in piglets at weaning showed that there were significant growth performance responses in the presence of DON contaminated feeds. Piglets consuming feeds without mycotoxin inhibitor had a lower weight gain and feed intake than those on feed containing mycotoxin inhibitors. However, the digestibility of nitrogen, calcium and phosphorus – as measured by their capacity to be absorbed, retained and excreted – unexpectedly failed to reflect the variation in growth performance.

Table of contents

List of tables

1 Objectives

1.1 Main objectives

Excerpt main objectives from detailed protocol (Schedule A).

Activity 2. The main objective of the project was to determine the impact of mycotoxin deoxynivalenol (DON) on susceptibility of pigs to infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2).

Activity 3. To evaluate the efficacy of feed additives in attenuating the toxicity of grains contaminated by mycotoxins and mitigating the negative effect of mycotoxins on the health (immune response) and performance of piglets.

1.2 Specific objectives

Excerpt specific objectives from detailed protocol (Schedule A).

Activity 2.

Objectives 1

Determine *in vitro* impact of DON on PRRSV (A) and PCV2 (B) replication in permissive cell line models,.

Objectives 2

Determine the impact of DON naturally contaminated feed on PRRSV (A) and PCV2 (B) infection, *in vivo*.

Activity 3. To determine the effects of mycotoxin contaminated feeds and mycotoxin inhibitor products:

a. on the retention of nutrients and growth performance in weaning piglets;

b. on barrier integrity as evaluated by absorption of mannitol and lactulose and histologic analysis of intestinal mucosa;

c. on digestibility of nutrients and on the relationship between this digestibility and digestive enzyme activities in intestinal mucosa; and

To develop quick indicators of mycotoxin exposition that would allow early detection of mycotoxicosis in live animals using a blood sample.

2 Project details

Include project details regarding methodology (i.e. experimental design, data collection, data analysis, findings).

Activity 2
Objectives 1A

Experimental design:
MARC-145 cells and porcine alveolar macrophages (PAMs) obtained from broncho-alveolar lavage of 42 to 70 day old PRRSV-negative piglets, known to be permissive to PRRSV were infected or not with PRRSV and treated with increasing concentrations of DON at 70, 140, 280, 560 and 1200 ng/ml and were incubated for 72 h. Cell viability and mortality were then determined. Medium alone was added as control. Viral replication has been also evaluated and

virus detection was performed using a commercial PRRSV real-time PCR diagnostic kit. Quantification of PRRSV was determined by comparing sample results to a standard curve based on the amount of serially diluted IAF-Klop PRRSV reference strain titrate after infection of MARC-145 cells and expressed as TCID50/ml. In order to evaluate the mechanism of action of DON on viral replication, expression of some antiviral and proinflammatory cytokines mRNA was evaluated by qPCR and triggering of apoptosis was also assessed by detecting procaspase 3 activation with a specific fluorescent substrate of this enzyme.

Objective 1B

Experimental design
Newborn porcine tracheal cells (NPTr) known to be permissive to PCV2 were persistently infected or not with PCV2a or PCV2b and treated with increasing concentrations of DON at 70, 140, 280, 560 and 1200 ng/ml and were incubated for 72 h. Cell viability and mortality were then determined. Medium alone was added as control. Viral replication has been also evaluated and virus detection was performed using a custom real-time quantitative PCR. Quantification of PCV2 was determined by comparing sample results to a standard curve based on the amount of serially diluted PCV2a Stoon 1010 strain titrate after infection of NPTr cells and expressed as TCID50/ml. In order to evaluate mechanism of action of DON on viral replication, the expression of some antiviral cytokines mRNA was evaluated by qPCR.

Objective 2a

Experimental design
Thirty (30) commercial piglets, negative for principal porcine pathogens were purchased locally at 4 weeks of age. After one week of acclimation, with a commercial ration, piglets were randomly divided to three experimental groups of 10 animals based on DON content of served diets (0, 1.5 and 3.5 PPM of DON/Kg), they wre fed these rations for 2 weeks. The day prior viral challenge, all pigs were weighed and blood sampled for viral quantification and antibody evaluation. All experimental groups were further subdivided into groups of six (6) pigs and were inoculated with 1 ml of $1.6x10^4$ $TCID_{50}$ PRRSV intra-muscularly and 1 ml of the same inoculums in each nostril. The remaining pigs (control) were sham-inoculated and received the same quantity of PBS. Pigs were monitored daily for temperature, weight and clinical signs for 21 days. Half pigs were euthanized at day 21 post-challenge, remaining pigs were sacrificed on day 22. Blood was collected at day 3, 6, 9, 13 and 21 and serum tested for PRRSV by real-time PCR. During necropsy, sections of lung and assorted lymph nodes were collected for formalin fixation and fresh tissue were stored at -20

Objective 2b

Experimental design
Thirty (30) commercial piglets, negative for principal porcine pathogens were purchased locally. In order to minimize the transfer of maternal-derived antibodies, piglets were weaned early on day 7 and fed milk replacer for 2 weeks. At 3 weeks of age, piglets were randomly divided into three experimental groups of 10 animals and fed DON contaminated diets (0, 1.5 and 3.5 PPM of DON/Kg) for 3 weeks. The day prior inoculation, all pigs were weighed and blood sampled for viral quantification and antibody evaluation. All experimental groups were further subdivided in groups of 6 pigs and were inoculated with 1ml of $1x10^5$ $TCID_{50}$ PCV2b intra-muscularly and 0.5 ml of the same inoculum in each nostril. The remaining pigs were sham-inoculated and received the same quantity of buffer (PBS) used to dilute viral preparation. Pigs were then monitored daily for temperature, weight and clinical signs for 14 days and weekly until day 30. Half pigs were euthanized at day 30 pi, remaining pigs were sacrificed at day 31. Blood was collected at day 9, 20, and 30 and serum tested for PCV2 by real-time PCR. At necropsy, sections of lung and assorted lymph nodes were collected for formalin fixation and fresh tissue storage at -20.

Activity 3

Animals and treatments: For this experiment, a total of 60 castrated weaning piglets (~ 6 kg, 20 days of age) were provided by a commercial farm. Piglets were housed in a pen (4 piglets per pen) for 5 days post-weaning. For these first five days, the piglets were fed with commercial post-weaning diets according to the feeding program recommended by the feed supplier.

After the 5 adaptation days, the 60 piglets were housed individually in pens for the following 7 days. The piglets were distributed in six treatments: a positive control group (<0.5 ppm DON), a negative control group, (4 ppm DON, naturally contaminated) and we measured the effects of four additives available commercially on the Canadian market. Four groups were thus fed with the negative contol diet and supplemented with one of four different feed additives (mycotoxin inhibitor products) selected according to their specific activities and characteristics:

1- Integral (Alltech)
2- Biofix (Biomin)
3- MXM (Jefo)
4- Defusion (Akey)

During the experiment, feed and water were provided *ad libitum* through the 7-day experimental period. Piglets were weighed on days 0 and 7 of the experiment. Feed intake was recorded daily. The basal diet was wheat, corn and soybean meal formulated according to NRC requirements (1998). For the control diet, non-contaminated wheat was used and naturally contaminated wheat was added so as to reach approximately 4 ppm of DON in the contaminated diets; DON was chosen because this mycotoxin is commonly detected in cereals and grains and is the most prevalent contaminating trichothecene in crop production in North America.

Table 1. Commercial mycotoxin inhibitor products incorporated into the experimental diets

Mycotoxin inhibitor products	Composition of mycotoxin inhibitor products	Contributing companies
Integral®	Yeast cell wall	Alltech, Lexington, KY
Biofix Plus®	Yeast cell wall, natural microbials and diatomaceous earth (clay)	Bio Min, Saint Antonio, TX
MXM®	Aluminosilicate	Jefo, Saint Hyacinthe, QC
Defusion®	Blend of preservatives, antioxidants, amino acids, and direct-fed microbials	Akey, Lewisburg, OH

Table 2. Composition of basal diets

Ingredients	%
Wheat (contaminated or not with DON)	33.0
Corn	29.75
Soybean meal	22.0
Hamlet Soy Protein HP300	5

Whey powder	6
Others[a]	4.25

[a]Calcium phosphate dibasic 1.85, Limestone 1.00, Salt 0.50, L-Lysine 0.25, DL-Methionine 0.07, Choline Chloride 0.08, Vitamin and mineral premix 0.50.

Table 3. Analyzed nutrient composition of experimental diets

Food composition	Positive control	Negative control	Integral	Biofix	MXM	Defusion
Fat (%)	2.70	2.83	2.87	2.91	2.98	2.93
Ash (%)	7.17	7.12	7.31	7.43	7.51	7.75
Nitrogen (%)	3.60	3.93	3.89	3.98	3.90	3.93
Calcium (%)	1.06	1.03	1.11	1.14	1.15	1.26
Phosphorus (%)	0.94	0.96	0.98	1.01	1.00	1.00
Energy (kcal)	4254.56	4292.51	4262.96	4274.60	4264.62	4257.70

After these 7 days, the piglets were transferred to metabolic crates for an adaptation period of 2 days followed by a period of 5 days for the collection of urine and feces in order to determine total digestibility of energy, nitrogen, ash, fat, Ca and P. Feces were collected by a bag stuck around the anus according to the method of van Kleef et al., (1994). Total urine was collected via funnels underneath the crates and transferred to an amber glass recipient containing HCl to reduce nitrogen loss. All feces and urine were weighed every day, and then frozen at -20°C.

On the morning following the collection period, a solution of mannitol (50 mg/kg body weight) and lactulose (500 mg/kg body weight) in 20 ml distilled water was administered. The urine was collected for the next 24 hours for the determination of mannitol and lactulose. Mannitol is absorbed by trans-cellular route of epithelial villi whereas lactulose traverses the intestinal wall by means of a para-cellular pathway via the intercellular tight junction of epithelial crypt. The lactulose : mannitol ratio is known as a reliable method to evaluate the intestinal permeability and mucosal barrier function.

At the end of urine collection, piglets were first sedated (*i.m* injection of xylazine-ketamine), then euthanized by IV injection of pentoparbital (120 mg/kg). The entire small intestine was quickly removed. Five cm from the middle section of the jejunum and ileum were removed for histological analysis (villi height and crypt depth). Histological analysis was used to assess the impact of mycotoxins on the general physiology of the intestinal mucosa: a reduction in the height of the villi associated with an increased crypt depth suggests a disruption of the physiology of the mucosa. The adjacent 30 cm were used for the determination of enzymatic activities (sucrase, lactase and animopeptidase N). The 30 cm intestinal segments were rinsed with ice-cold saline. Then, the mucosa was scraped off with a glass slide and frozen individually in liquid nitrogen and stored at -80°C until analysis. The enzyme activity of the intestinal mucosa is an important part of digestion and decrease of these activities may reduce the digestive capacity of the animal and thus overall growth.

Determination of mycotoxins in serum: For the evaluation of DON and its metabolites in serum, blood samples were collected on days 7 and 13 and DON and its metabolite epoxy-DON (DOM-1) concentration were assessed by HPLC using immune-affinity column (IAC), as described below. Briefly, 1 ml acetate buffer and 60 µl ß-glucuronidase are added to 1.5 ml of serum and incubated for 16h in 37°C. After filtration, 0.5 ml of the solution is added into IAC and washed with methanol. Elute is evaporated under nitrogen and resuspended in mobile phase (12% ACN and 88% H_2O) before HPLC analysis.

Statistical analysis. In this study, the experimental unit is the piglet. The impact of dietary treatments on measured variables during the experimental period will be evaluated statistically using the SAS mixed model. A priori contrasts were defined to determine the differences between the positive control and the negative control (effect of DON), the negative control and treatment 3 (effect of Integral), the negative control and treatment 4 (effect of Biofix), the negative control and treatment 5 (effect of MXM) and the negative control and treatment 6 (effect of Defusion).

3 Funded project participants

Indicate names and organizations of funded scientists and participants and their respective roles. Add additional rows for scientists (or participants) as needed.

Table 1. List of names and organizations of funded scientists and participants

Funded Scientist or Participant		Organization
Name	**Role**	
Bich Van Le Thanh	Graduated Student	Université Laval
Vicente Pinilla	Graduated Student	Université de Montréal

4 Non-funded project participants

Indicate names and organizations of non-funded scientists and participants and their respective roles. Add additional rows for scientists (or participants) as needed. Note: Include name of Project Leader.

Table 2. List of names and organizations of non-funded scientists and participants

Non-funded Scientist or Participant		Organization
Name	**Role**	
Christian Savard	Postdoctoral fellow	Université de Montréal
Frédéric Guay	Professor	Université Laval
Younes Chorfi	Professor	Université de Montréal
Martin Lessard	Researcher	AAC
Carl Gagnon	Professor	Université de Montréal
Mariela Segura	Associate professor	Université de Montréal

5 Project results

All Project 'Expected Outputs / Deliverables' have been entered into Table 3. Please complete all un-shaded blank cells in Table 3:

Table 3. Detailed project results

Expected Outputs/Deliverables (Refer to Schedule C of the agreement)	**Status of Activity** Completed OR Not Completed	**Significant achievements**	**Identify any major issues or variance between planned and actual deliverables and the budget impact**	**Corrective Action(s)**	**% Completed** (0% to 100%)
ACTIVITY : 1- Effects of vomitoxin (DON) contaminated feed on susceptibility to viral infections (circovirus, PRRSV) and on immune response in piglets (#2)					
EXPECTED OUTPUTS/DELIVERABLES: Expected results are that DON affects PRRSV and PCV2 replication in their respective immortal permissive cell lines (MARC-145 cells and SJPL for PRRSV; PK15 and NPTr for PCV2) and cell death by apoptosis.					
	Completed				
EXPECTED OUTPUTS/DELIVERABLES: Expected results are that DON: impairs functional properties of immune-competent cells and decreases animal resistance to PRRSV and PCV2 infections. Therefore, in piglets fed with a diet contaminated with DON, the innate and acquired immune response to PCV and PRRSV is negatively affected, as assessed by their effects on functional properties of PBMC. This study will also contribute to a better understanding of the effect of DON on animal health and immune response to viral infections caused by PRRSV and PCV2 and lead to recommendations to producers with a view to improve business profitability.					
	Completed				
EXPECTED OUTPUTS/DELIVERABLES: Expected results are that in piglets fed with a diet contaminated with DON, clinical lesions associated with PCV2 and PRSSV will be more severe, reflecting a greater effect of these infections and therefore a possible reduction in growth performance.					
	Completed				
EXPECTED OUTPUTS/DELIVERABLES: This study will also contribute to a better understanding of the effect of DON on animal health and immune response to viral infections caused by PRRSV and PCV2 and lead to recommendations to producers with a view to improve business profitability.					

Expected Outputs/Deliverables (Refer to Schedule C of the agreement)	**Status of Activity** Completed OR Not Completed	**Significant achievements**	**Identify any major issues or variance between planned and actual deliverables and the budget impact**	**Corrective Action(s)**	**% Completed** (0% to 100%)
	Completed				
ACTIVITY : 3- Evaluate the effects of mycotoxin contaminated feeds and feed additive supplements on: 1) barrier integrity and function of the intestine, including digestive enzyme activities; 2) digestibility and retention of nutrients, and 3) metabolism circulation and excretion of DON (#3)					
EXPECTED OUTPUTS/DELIVERABLES: Expected results are that DON: reduces the integrity and barrier function of the mucosa and that feed additive supplements partially restore the intestinal barrier.					
	Completed	Histological examination of the mucosa and mannitol _lactoluse analyzes have been completed.	.		100
EXPECTED OUTPUTS/DELIVERABLES: Expected results are that DON: reduces the digestibility and retention of nutrients and digestive enzyme activities, and that feed supplements improve global retention of nutrients.					
	Completed	Laboratory analysis on feed, feces, urine and mucosa samples have be done (completed in August 2012). Statistical analysis has been done on growth			100

Expected Outputs/Deliverables (Refer to Schedule C of the agreement)	**Status of Activity** Completed OR Not Completed	**Significant achievements**	**Identify any major issues or variance between planned and actual deliverables and the budget impact**	**Corrective Action(s)**	**% Completed** (0% to 100%)
		performance, nutrient balance, and digestive enzyme activities (completed in November 2012).			
EXPECTED OUTPUTS/DELIVERABLES: Expected results are that DON and its metabolites (in serum and excreted in urine) are easily detectable and are good indicators of exposure of animals to mycotoxins.					
Expected results are that DON and its metabolites (in serum and excreted in urine) are easily detectable and are good indicators of exposure of animals to mycotoxins.	Competed	DON was analyzed only in the serum. After discussion we decided to use only the serum as blood is more easily accessible than urine in commercial farm conditions.	.		100
EXPECTED OUTPUTS/DELIVERABLES: This study will also contribute to a better understanding of the effect of DON on digestibility and retention of nutrients so that adjustments to the supply of nutrients needed to maintain growth and performance can be made leading to recommendations to producers with a view to improve business profitability.					
	Completed	This project has shown that DON altered the digestibility and utilization of nutrients. However, we cannot			100

Expected Outputs/Deliverables (Refer to Schedule C of the agreement)	**Status of Activity** Completed OR Not Completed	**Significant achievements**	**Identify any major issues or variance between planned and actual deliverables and the budget impact**	**Corrective Action(s)**	**% Completed** (0% to 100%)
		currently recommend a specific modification of nutrient requirements in the presence of DON. A second part of this project was presented to the Swine cluster II.			

Project results (continued)

Discussion of Project results - What worked? What didn't work? What results were especially interesting? What new questions (and research) might result from this project?

Activity 2
Objective 1A

Fig 1:

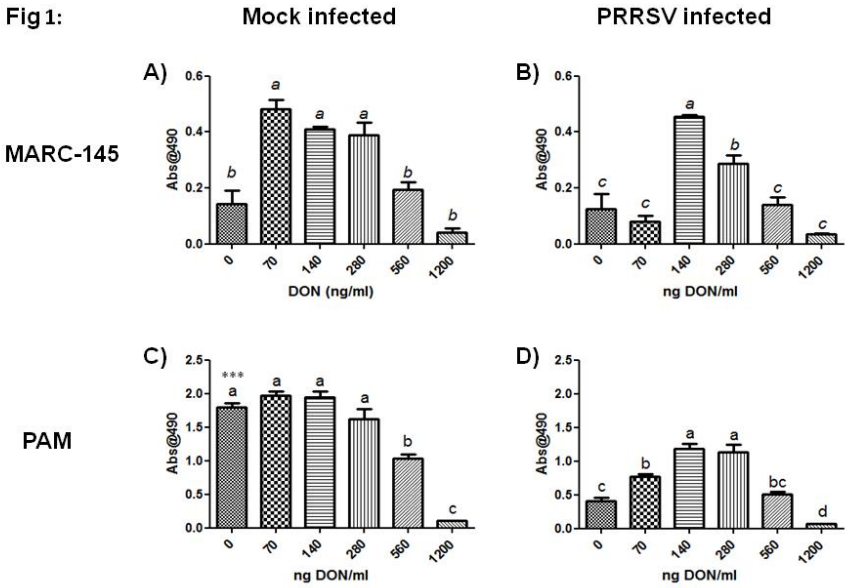

Figure1. Effect of DON on cell viability following PRRSV infection. Mock infected (A,C) or PRRSV (IAF-Klop) infected (B,D) MARC-145 (A,B) or PAM (C,D) cells were simultaneously treated with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Cells viability was measured with CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) 72 h following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$). *** compare non infected and infected cells without DON ($P < 0.,001$). Results are representative of three independent experiments for each cell types.

Fig 2:

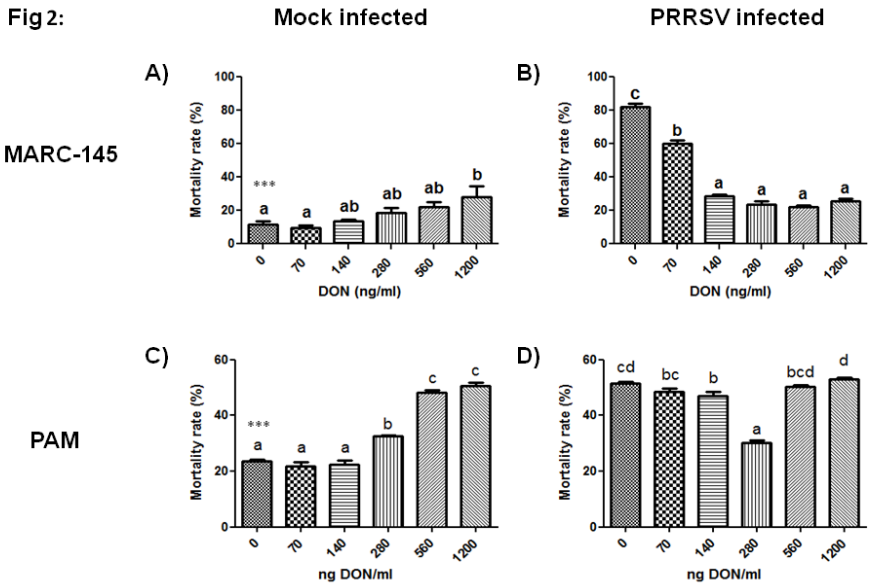

Figure 2. Effect of DON on cell mortality following PRRSV infection. Mock infected (A,C) or PRRSV (IAF-Klop) infected (B,D) MARC-145 (A,B) or PAM (C,D) cells were treated simulteanously with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Cells mortality was evaluated by LDH release, with CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) 72 h following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$). *** compare non-infected and infected cells without DON ($P < 0.001$). Results are representative of three independant experiments for each cell types.

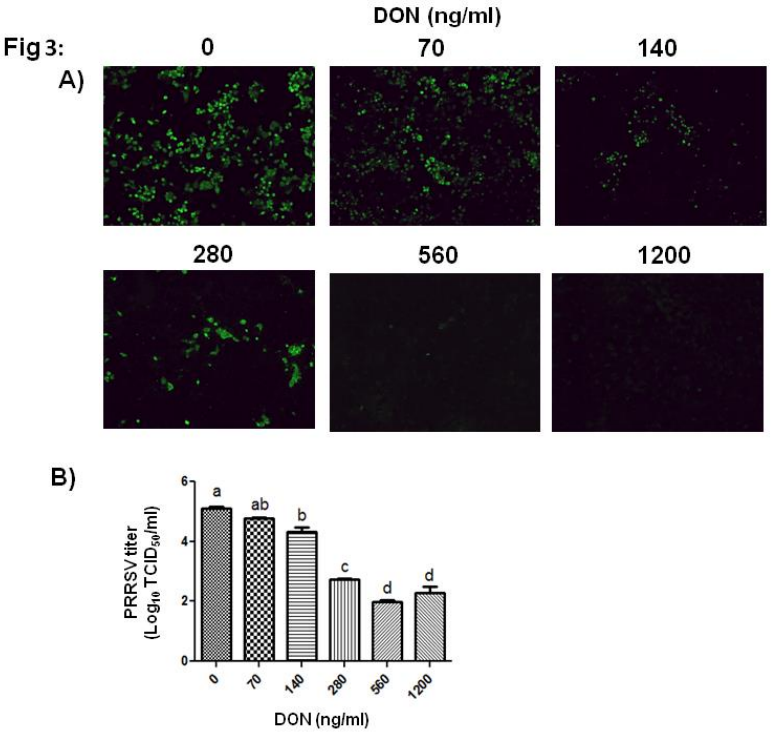

Figure 3. Effect of DON on PRRSV replication. PRRSV (IAF-KLOP) infected MARC-145 cells were simulteanously treated with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml) for 72 h. A) PRRSV viral titers were measured by immunofluorescence with a PRRSV N-specific antibody. B) PRRSV infected PAM cells were simulteanously treated with the same concentrations of DON for 72 h. PRRSV viral titers were measured, in triplicatas, by qPCR. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$).

Fig 4:

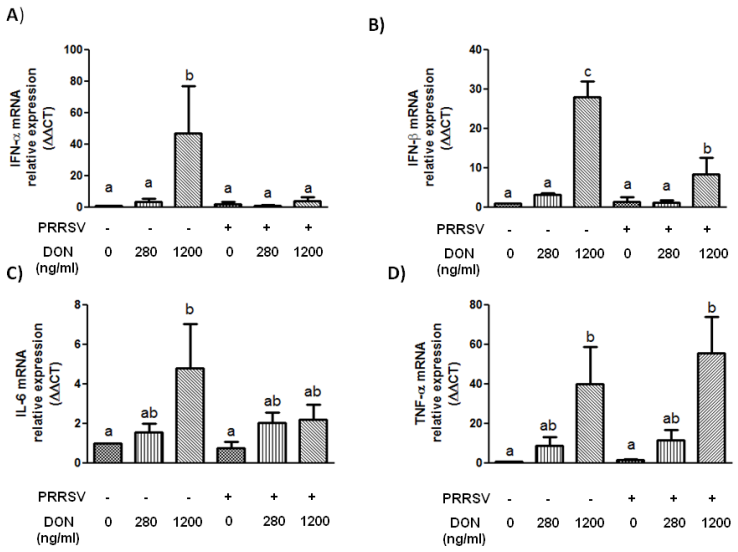

Figure 4. Cytokine mRNA expression by qPCR. Mock infected or PRRSV (IAF-KLOP) infected PAM cells from 3 different pigs were treated with increasing concentrations of DON mycotoxin (0, 280, or 1200 ng/ml) for 3 h. The relative mRNA expression of some antiviral IFN-α (A) and IFN-β (B) and pro-inflammatory genes IL-6 (C) and TNF-α (D) genes were measured by quantitative PCR. The data is expressed in ΔΔCt using the β_2-microglobulin gene as the normalizing gene and the noninfected cells as the calibrator reference. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$).

Fig 5:

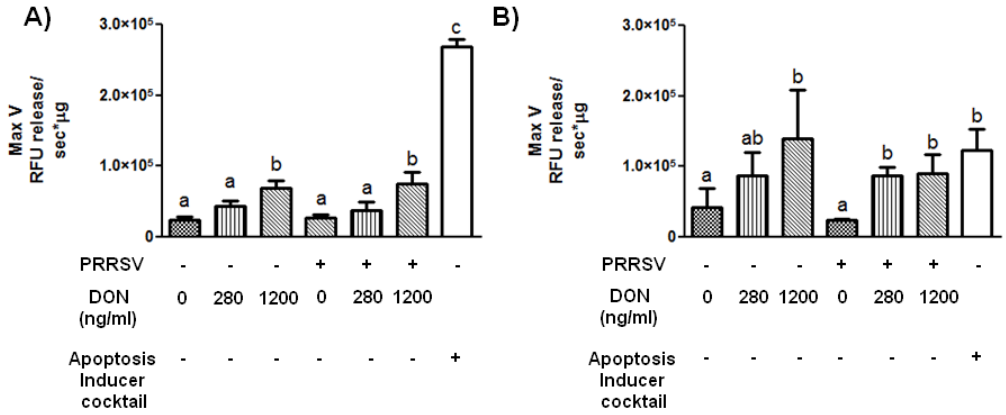

Figure 5. Caspase 3 activation. Mock infected or PRRSV (IAF-KLOP) infected PAM cells, from 3 different pigs, were treated with increasing concentrations of DON mycotoxin (0, 280, or 1200 ng/ml) for 3 h (A) or 6 h (B). As positive control, cells were treated with a cocktail of known apoptosis inductor containing 20 µg/ml of cycloheximide, 0.5 µg/ml actinomycin D and 2 µg/ml vinblastin sulfate. The results are expressed as relative fluorescence released (relative fluorescence units or RFU) per second per µg of cell lysates. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$).

Data analysis

Results showed that DON concentrations of 560 ng/ml or higher were significantly detrimental to the survival of MARC-145 and PAM cells at 72 h (Fig 1A,C). For MARC-145 cells (Fig 1A), a decrease of absorbance in non-treated cells was observed compared to DON-treated cells, even

though cell morphology appeared to be normal under light microscopy suggesting a nonlinearity of the assay for this cell type. Viability of cells infected with PRRSV has also been evaluated with aforementioned conditions. The viability of noninfected (Fig 1A, 0 ng/ml DON) and infected (Fig 1B, 0 ng/ml DON) MARC-145 cells, not treated with DON were similar, even if cytopathic effect was observed under light microscopy However, a significant ($p < 0,001$) reduction of viable cells was found in PRRSV infected PAM cells (Fig. 1D, 0 ng/ml DON) without DON due to the virus cytopathic effect compared to noninfected cells (Fig 1C, 0ng/ml DON). In contrast, there was a significant increase of viable cells at DON concentrations within 140 to 280 ng/ml and 70 to 280 ng/ml ranges for MARC-145 (Fig 1B) and PAM (Fig 1D) cells, respectively. These results indicate that DON sub-toxic concentrations could significantly reduce the cytopathic effect caused by PRRSV. As for noninfected cells, DON concentrations higher than 560 ng/ml decreased the number of viable cells.

In order to solve the nonlinearity of viability assay for the MARC-145 cells and to confirm the other results of cell viability, cell mortality was evaluated using LDH release as indicator of cell membrane integrity, 72 h following the same DON treatment. As for the viability assay, only high concentrations of DON increased mortality of MARC-145 cells (Fig 2A). PAM cells appeared to be more sensitive to DON than MARC-145 cells, as concentrations of 280 ng/ml and higher of the toxin were sufficient to significantly increase LDH release by these cells (Fig 2C).
As expected, PRRSV infected MARC-145 (Fig 2B, 0 ng/ml DON) and PAM (Fig 2D, 0 ng/ml DON) cells without DON had higher ($p<0,001$) mortality rate than their noninfected counterparts (Fig 2A,CD, 0 ng/ml DON), because PRRSV is a cytopathic virus. Exposure to low concentration of DON progressively decreased mortality of infected MARC-145 cells (Fig 2B) starting at 70 ng/ml and reaching basal level at 280 ng/ml or higher. DON concentration required to decrease mortality of infected PAM cell was slightly higher than for MARC-145 cells, at 140 ng/ml and 280 ng/ml (Fig 2D). Since PAM cells appeared to be more sensitive to DON toxicity than MARC-145 cells, the mortality rate increased again with concentrations over 280 ng/ml. These results confirm those obtained with cell viability, that sub-toxic concentrations of DON could significantly reduce PRRSV cytopathic effect.

Our previous results have shown that sub-toxic concentrations of DON significantly decreased mortality caused by PRRSV. To test DON effect on PRRSV replication, immunofluorescence experiment was performed to detect PRRSV N antigen in MARC-145 infected cells treated with DON. Results showed that the staining of PRRSV gradually decreased starting at 140 ng/ml of DON until complete disappearance of labeling at 560 ng/ml or higher (Fig 3A). In order to confirm this result, the experiment was performed in PAM cells, the natural target cell of the virus. Cell pellet and supernatant were subjected to a quantitative PCR assay to determine virus titer. Results demonstrated that PRRSV titer gradually decreased starting at 140 ng/ml of DON until reaching basal level at 280 ng/ml or higher (Fig 3B). Taken all together, these results suggest that DON could inhibit PRRSV replication in a dose dependant manner, which may explain the decrease in cell mortality caused by DON in infected cells, since the replication of virus was affected.

Relative mRNA expression of some antiviral (IFN-α and IFN-β) and pro-inflammatory (IL-6 and TNF-α) genes were measured in PAM cells following exposure to increasing doses of DON (0, 280 and 1200 ng/ml). Relative mRNA expression of the same genes were also measured on PRRSV infected PAM cells exposed to same concentrations of DON (Fig 4). Results demonstrated that the relative expression of all tested cytokines mRNA were upregulated only with the highest DON dose, 3 h following exposition to DON alone. However, the increased expression of antiviral mRNAs (Fig 4A,B) was significantly offset by PRRSV infection, which is not the case for pro-inflammatory mRNA such as IL-6 and TNF-□□(Fig 4C,D).

Exposure to DON is also known to induce apoptosis, which could also contribute to the inhibitory effect of DON on PRRSV. Apoptosis is characterized by morphological and biochemical changes, including caspases activation. In order to evaluate apoptosis triggering, caspase-3 like activity was measured. Results showed that caspase-3 activation was increased in a dose dependant

manner, 3 h and 6 h after DON exposition (Fig 5A and B). At 3 h pi, only the highest concentration of DON has increased significantly caspase-3 activity however at 6 h pi, this activity has significantly increased for both DON concentrations As for pro-inflammatory genes, the increase of caspase-3 activation was not significantly changed by PRRSV infection.

Objective 1B

Fig. 1

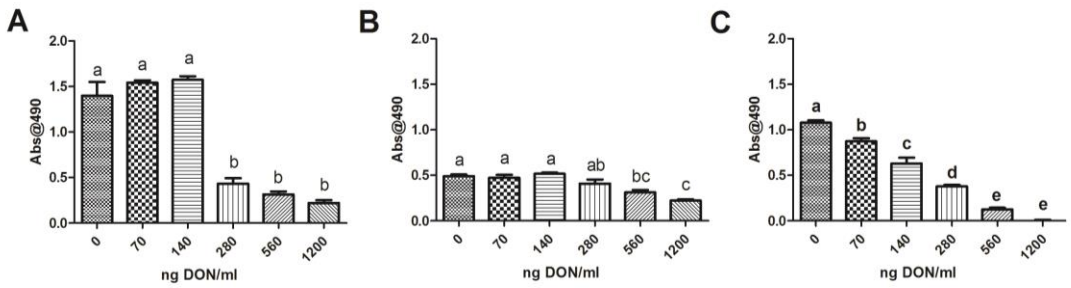

Figure1. Effect of DON on cell viability following PCV2 infection. Mock infected (A) or PCV2a (Stoon 1010) (B) or PCV2b (FMV-06-0732) (C) persistently infected NPTr cells were simultaneously treated with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Cells viability was measured with CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) 72 h following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$). Results are representative of two independent experiments for each cell types.

Fig. 2:

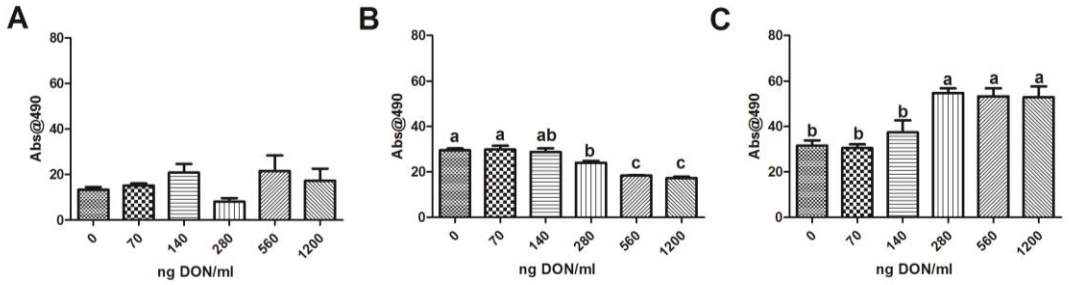

Figure 2. Effect of DON on cell mortality following PCV2 infection. Mock infected (A) or PCV2a (Stoon 1010) (B) or PCV2b (FMV-06-0732) (C) persistently infected NPTr cells were treated simultaneously with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Cells mortality was evaluated by LDH release, with CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) 72 h following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$). Results are representative of two independant experiments for each cell types.

Fig 3:

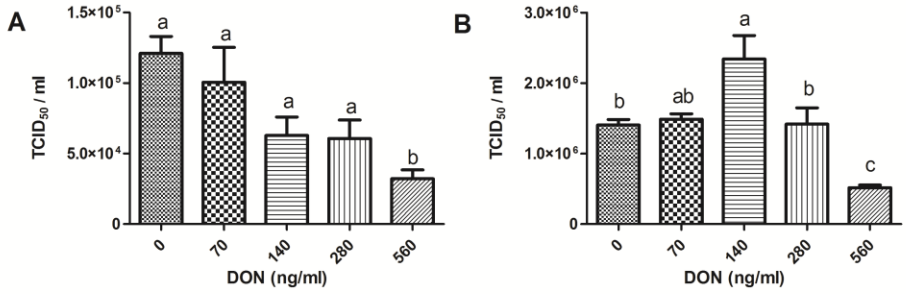

Figure 3. Effect of DON on PCV2 replication. PCV2a (Stoon-1010) (A) or PCV2b (FMV-06-0732) (B) persistently infected NPTr cells were simultaneously treated with increasing concentrations of DON (0, 70, 140, 280 or 560 ng/ml) for 72 h. PCV2 viral titers were measured, in triplicatas, by quantitative PCR. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$).

Fig. 4:

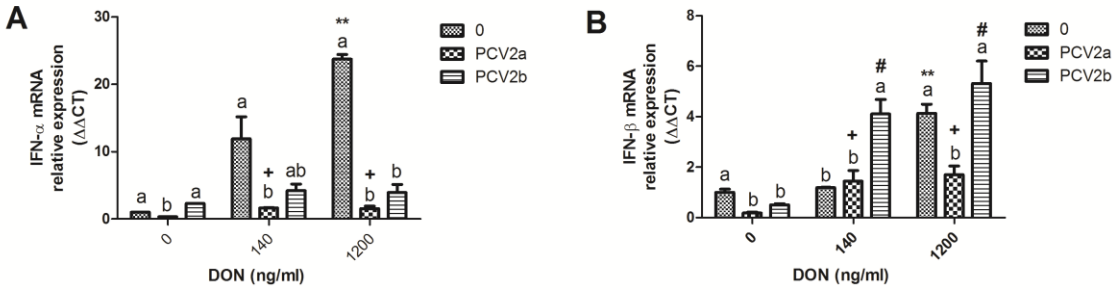

Figure 4. Cytokine mRNA expression by qPCR. Mock infected or PCV2a (Stoon-1010) or PCV2b (FMV-06-0732) persistently infected NPTr cells were treated with increasing concentrations of DON mycotoxin (0, 140, or 1200 ng/ml) for 3 h. The relative mRNA expression of some antiviral IFN-α (A) and IFN-β (B) genes were measured by quantitative PCR. The data is expressed in ΔΔCt using the β_2-microglobulin gene as normalizing gene and the noninfected cells as calibrator reference. Data labeled with superscripts of different letters indicate significant difference between data within the same DON concentration ($P < 0.05$). ** compare effect of DON concentration in non-infected cells ($P < 0.01$). + compare effect of DON concentration in PCV2a persistently infected cells ($P < 0.05$). # compare effect of DON concentration in PCV2b persistently infected cells ($P < 0.05$).

Data analysis

Results showed that DON concentrations of 280 ng/ml or higher were significantly detrimental to the survival of noninfected NPTr cells at 72 h (Fig 1A). These results also demonstrate that not treated NPTr cells infected with either PCV2a or PCV2b have a lower viability compared to uninfected cells (Fig. 1B,C,A, 0 ng/ml DON, respectively). DON have a genotype dependant effect on PCV2 infected NPTr cells. In fact, DON has no significant effect on cell viability of PCV2a infected NPTr cells (Fig 1B). Conversely, DON has a dose dependent effect on viability of PCV2b infected NPTr cells (Fig 1C). Even sub-toxic concentrations of DON gradually decreased viability of PCV2b infected NPTr cells. As for uninfected cells, concentrations above 280 ng/ml of DON significantly decreased viability of PCV2-infected cells regardless of genotype. Taken

together, these results suggest that DON could potentially increase replication of PCV2b but have a limited impact on replication of PCV2a.

In order to confirm results of cell viability, cell mortality was also evaluated using LDH release as indicator of cell membrane integrity, 72 h following DON treatment. Unlike cell viability, DON has not had a significant effect on cell mortality of uninfected cells, at the concentration used (Fig 2A). These data indicate that DON has the capacity to significantly reduce cell viability without causing loss of membrane integrity, an indication of cell mortality. However, cell mortality results confirm those obtained in cell viability since the mortality of untreated PCV2 infected NPTr cells is higher than these of uninfected cells (Fig. 1B,C,A, 0 ng/ml DON, respectively). These results indicate that although PCV2 not cause visible cytopathic effect, it still has a negative impact on cell survival. DON has had a significant impact on the mortality of NPTr cells persistently infected with PCV2. The observed effect was dependant of the DON concentration and of the PCV2 genotype. In PCV2a persistently infected cells, the mortality was slightly decreased when cells were treated with 280 ng/ml or higher of DON (Fig. 2B), while for the same DON concentrations, mortality of PCV2b persistently infected cells were strongly increased (Fig 2C). Taken together these results suggest that replication of PCV2b could be enhanced by the addition of DON, even at sub-toxic concentrations while those of PCV2a would be rather reduced.
In order to verify the precedent hypothesis, PCV2 replication was evaluated by quantitative PCR. Results showed that the amount of virus present in PCV2a infected cells has decreased gradually according to DON concentration, until to a concentration of 560 ng / ml where it has significantly decreased (Fig 3A). Data on PCV2b infected cells were slightly different since an increase in the amount of virus present in the PCV2-infected cells was observed at a concentration of 140 ng / ml of DON (Fig 3B). However, as for the PCV2a infected cells, high concentration of DON 560 ng/ml significantly reduced the presence of viruses in PCV2b infected cells. These results confirm the previously emitted hypothesis that replication of PCV2b could be enhanced by the addition of DON while those of PCV2a would be rather reduced. However high concentration of DON seems to strongly reduce viral replication of PCV2 regardless of genotypes, potentially by affecting cell survival.
Since DON is known to stimulate interferons mRNA expression and that interferons have been shown to be capable of increasing PCV replication, the relative mRNA expression of type I interferons (IFN-α and IFN-β) were measured in NPTr cells following exposure to increasing doses of DON (0, 280 and 1200 ng/ml). Relative mRNA expression of the same genes was also measured on PCV2a and PCV2b persistently infected NPTr cells exposed to same concentrations of DON. Results demonstrated that the relative expression of both interferons mRNA were upregulated only with the highest DON dose, 3 h following exposition to DON alone (Fig 4A,B). However, the increased expression of IFN-α mRNAs (Fig 4A) was significantly reduced in PCV2a and PCV2b infected cells. IFN-β mRNA expression in PCV2 infected cells was completed different depending of PCV2 genotypes (Fig 4B). In fact at 140ng/ml of DON the IFN-β mRNA expression in uninfected and PCV2a infected cells were similar, but higher in PCV2b infected cells.

Objective 2a

Data collection.

Fig. 1:

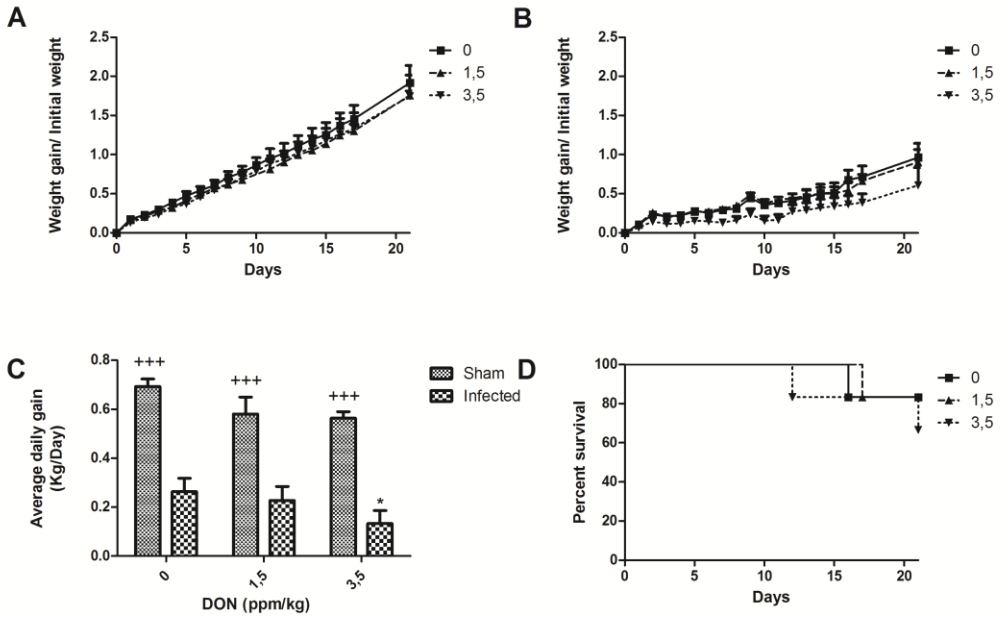

Figure 1. Effect of DON naturally contaminated diets on piglet's growth following PRRSV infection.Groups of piglets (10) were fed DON naturally contaminated diets (1.5 and 3.5 ppm/kg of feed) for 2 weeks. Other group of piglets received a noncontaminated diet for the same period. One portion of piglets (6) was PRRSV infected (B) and remaining piglets (4) were sham infected with PBS (A). Piglets were weighted daily during the infection period. A growth curve was obtained using a ratio between weight gain and initial weight for each weighing (A,B). Average daily gain was calculated by dividing total weight gain by the number of days of the study (C). Kaplan-Meier survival curve with end points piglet death (D).

Fig. 2:

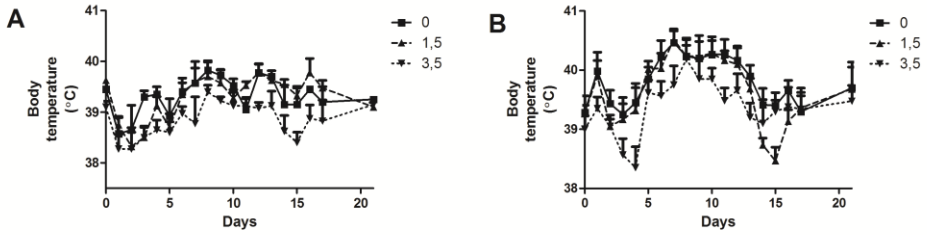

Figure 2. Time course of rectal body temperature during infection. noninfected (A) and infected (B) pigs were monitored daily for rectal body temperature during the infection period.

Fig 3

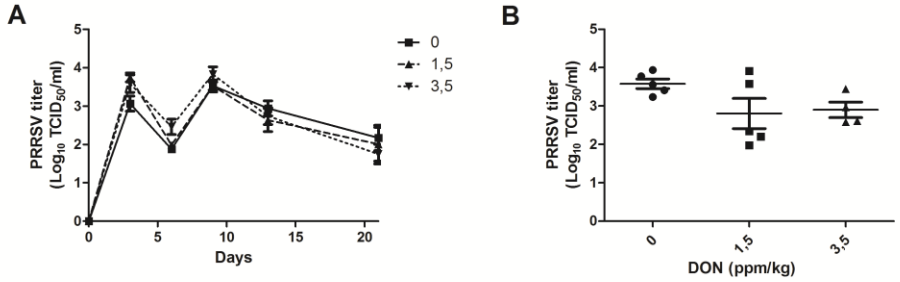

Figure 3. Effect of DON naturally contaminated diets on PRRSV viremia and viral load in the lungs. Groups of piglets (10) were fed DON naturally contaminated diets (1.5 and 3.5 ppm/kg of feed) for 2 weeks. Other group of piglets received a noncontaminated diet for the same period. One portion of piglets (6) was PRRSV infected and remaining piglets (4) were sham infected with PBS. Blood was collected at day 3, 6, 9, 13, and 21 and serum tested for presence of PRRSV RNA by real-time qPCR (A). At necropsy, sections of lung were collected to determine the pulmonary viral load by qPCR (B). Data are expressed in $TCID_{50}$/ml.

Fig. 4:

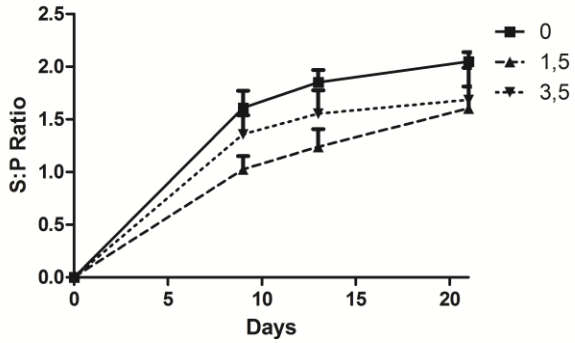

Figure 4. Effect of DON naturally contaminated diets on PRRSV specific antibody response. Groups of piglets (10) were fed DON naturally contaminated diets (1.5 and 3.5 ppm/kg of feed) for 2 weeks. Other group of piglets received a noncontaminated diet for the same period. One portion of piglets (6) was PRRSV infected and remaining piglets (4) were sham infected with PBS. Blood was collected at day 3, 6, 9, 13, and 21 and serum tested for presence of specific PRRSV antibody with a commercial ELISA kit (HerdChek-PRRS®,IDEXX). Data are expressed in ratio of sample to positive.

Data analysis

Analysis of the growth performance as evaluated by weight gain shows that noninfected pigs had significantly higher weight than PRRSV infected pigs (Fig 1A,B) regardless of the DON treatment. The same conclusion can be drawn when the analysis is based on the overall average daily gain obtained over the entire duration of the study (Fig 1C). A severe weight loss was observed following infection with the PRRSV strain used in this study showing it high potential virulence. Mortality rate due to infection was relatively raised, being above 15% for all infected groups (Fig 1D). Only the highest concentration of DON (3.5 ppm) present in naturally contaminated feed had significant ($P < 0,05$) effect on the average daily gain of noninfected groups with a loss of approximately 19% of kg/day (Fig 1C). Same level of contamination in infected animals had more dramatic impact since the animals that had ingested the diet at 3.5 ppm had an average daily weight gain of 40% less than animals fed the control diet (Fig 1C). These data suggest that pigs fed diet with high mycotoxin content are more affected by PRRSV than those fed a “clean” diet.

The majority of infected pigs in uncontaminated group (5 out of 6) or 1.5 ppm DON / kg (4 out of 6) fed diet had fever (body temperature between 39.8 to 41.5) for prolonged periods (over 6 days) (Fig 2B). In contrast, no pig from infected group receiving the highest dose of DON had fever episode more than 6 days.

All experimentally infected pigs, regardless of DON contamination levels, developed an important viremia from day three post-challenge throughout the study (Fig 3A). Viral load in the lungs, taken at the end of the study, was also important in all experimentally infected animals (Fig 3B) that survived the infection. DON contamination appears to decrease PRRSV replication since viral load in the lungs were respectively five and six times lesser in pigs fed diet 1.5 ppm (*P*=0,0965) and 3.5 ppm (*P*=0,0195) of DON than pigs receiving uncontaminated diet (Fig 3B). DON had a limited impact on viremia (Fig 3A), regardless of DON treatment, which were similar between treated and untreated groups.

Full analysis of macroscopic lesions observed at necropsy is not yet completed. However, the lesions observed in animals receiving 1.5 ppm / kg of DON seemed less important than in other groups. Microscopic lesions analysis is about to be done, and will possibly confirm the observations recorded at necropsy.

As demonstrated by measuring serum specific antibodies to PRRSV, all experimentally infected animals, regardless of DON contamination, had seroconverted. The humoral response was significantly ($P < 0,05$) lower in the animals ingesting 1.5 ppm/kg of mycotoxin and nearly lower (P =0,0639) in animals fed 3.5 ppm/kg, when compared to animals fed uncontaminated diet (Fig 4).

Objective 2b

Data collection.

Fig. 1:

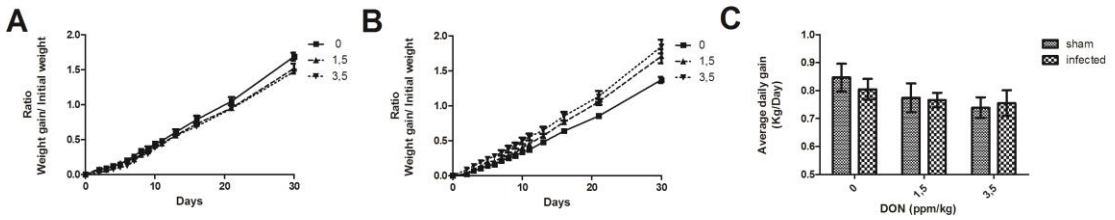

Figure1. Effect of DON naturally contaminated diets on piglet's growth following PCV2b infection.
Groups of piglets (10) were fed DON naturally contaminated diets (1.5 and 3.5 ppm/kg of feed) for 3 weeks. Other group of piglets received a non contaminated diet for the same period. One portion of piglets (6) were PCV2b infected (B) and remaining piglets (4) were sham infected with PBS (A). Piglets were weighted daily for 14 days and weekly until day 30 during the infection period. A growth curve was obtained using the ratio between weight gain and initial weight for each weighing (A,B). Moreover, the average daily gain was calculated by dividing the total weight gain on the number of days of the study (C).

Fig. 2:

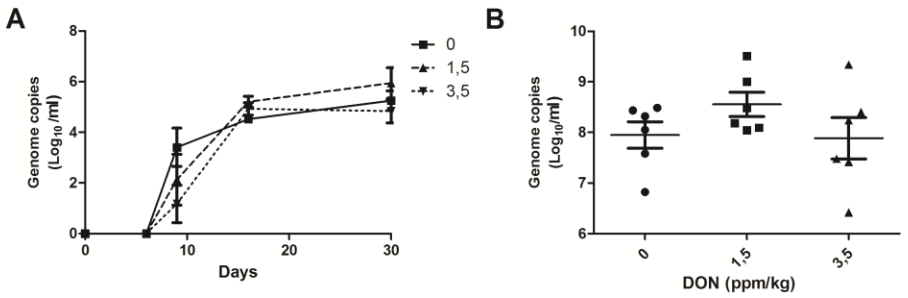

Figure2. Effect of DON naturally contaminated diets on PCV2b viremia and viral load in lungs.
Groups of piglets (10) were fed DON naturally contaminated diets (1.5 and 3.5 ppm/kg of feed) for 3 weeks. Other group of piglets received a non contaminated diet for the same period. One portion of piglets (6) was PCV2b infected and remaining piglets (4) were sham infected with PBS. Blood was collected at day 9, 16, and 30 and serum tested for presence of PCV2b DNA by real-time qPCR (A). At necropsy, sections of lung were collected to determine pulmonary viral load by qPCR (B). Data are expressed in genome copies of virus by ml.

Fig. 3:

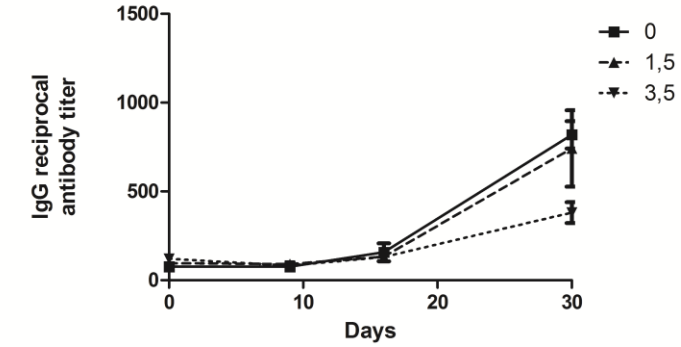

Figure3. Effect of DON naturally contaminated diets on PCV2b specific antibody response.
Groups of piglets (10) were fed DON naturally contaminated diets (1.5 and 3.5 ppm/kg of feed) for 3 weeks. Other group of piglets received a non contaminated diet for the same period. One portion of piglets (6) were PCV2b infected and remaining piglets (4) were sham infected with PBS. Blood was collected at day 9, 16, and 30 and serum tested for presence of specific PCV2b antibody with a commercial ELISA kit (INGEZIM CIRCO IgG).

Data analysis

Data analysis of noninfected pigs reveals that these animals had a slightly higher growth performance than PCV2b infected pigs (Fig 1A,B at 0 PPM/kg of DON). The same conclusion can be drawn when the analysis is based on the overall average daily gain obtained over the entire duration of the study (Fig 1C at 0 PPM/kg of DON). No severe weight loss was observed following infection with the PCV2b strain used in this study, one classical clinical sign of the disease associated to PCV2. The presence of DON in feed had a greater impact on growth performance than PCV2b infection. However, only the highest concentration of DON present in naturally contaminated feed had a slight effect on the average daily gain but not significant (P = 0.1291) loss of approximately 15% of kg/day (Fig 1C). DON had little impact on the growth of PCV2b infected pigs because the weight of piglets that received the contaminated diets, regardless of the dosage, was similar between infected and uninfected groups (Fig 1C). Although

these results should be interpreted with caution, the data suggest that pigs fed the diet without mycotoxin are more affected by PCV2b. Repetition of these results with more animals per group would be necessary to reach a definitive conclusion.

All experimentally infected pigs, regardless of DON contamination, developed an important viremia from day 9 post-infection that was maintained until the end of the experiment (Fig 2A). Viral load in the lungs, taken at the end of the study, was also important in all experimentally infected animals (Fig 2B). DON contamination at 1.5 ppm appears to increase slightly (P = 0.1205) the replication of PCV2 since piglets fed with this diet had a higher viremia (Fig 2A) and a viral load in the lungs four times greater than those that received uncontaminated diet (Fig 2B). Higher DON contamination at 3.5 ppm had limited effects on viremia (Fig 2A) and viral load in pig lungs (Fig 2B), these effects were similar between infected and noninfected groups.

As demonstrated by specific antibodies to PCV measured in the serum, all experimentally infected animals, regardless of DON contamination, has seroconverted. The humoral response at day 30 was significantly ($P < 0.05$) lower in animals ingesting the highest concentration of mycotoxin in the diet (Fig 3).

All data presented here, combined with the fact that no significant macroscopic and microscopic lesions were observed suggest that all PCV2b experimentally infected pigs included in this study had developed only subclinical infections.

Activity 3
1. Effects of DON and feed additives on nutrient disposition and growth performance

1.1. Growth performance

Average daily gain (ADG, g/day), average daily feed intake (ADFI, g/day) and feed efficiency (G:F) are used to evaluate growth performance in animals. Results presented in the figure below are on piglets under different dietary treatments for 14 days.

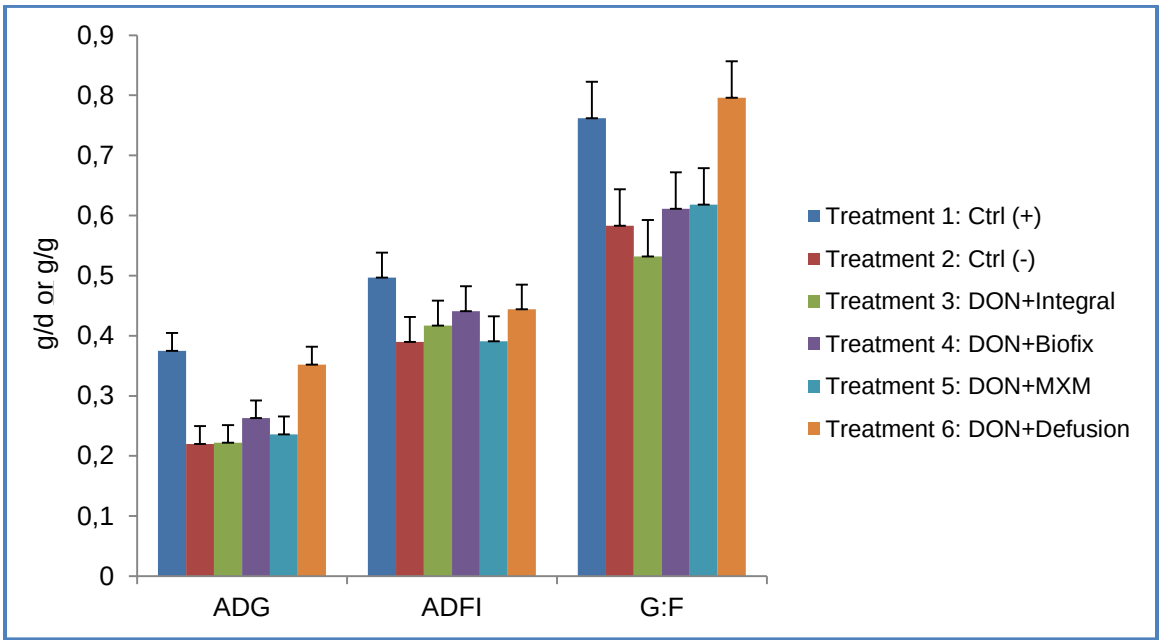

Figure 1. Effects of experimental diets containing concentrations of DON and feed additives on average daily gain, average daily feed intake and feed efficiency of pigs

Feeding the experimental diets over a period of 14 days did not lead to significant differences in ADG, ADFI and G:F ratio among treatment groups 2, 3, 4 and 5 (*P>0.05*).

Mean ADG of the piglets was significantly lower in treatment 2 than in treatments 1 (*P<0.0001*) and 6 (*p=0.0005*). Mean ADFI of the piglets in treatment 2 was not significantly different than in treatments 3 (*p=0.27*) and 5 (*P=0.98)*. However, mean ADFI was significantly lower in treatment 2 than in treatments 4, 6 and 1 (*P<0.05*).

During the experimental period, the mycotoxin inhibitor product in treatment 6 can apparently degrade DON or mitigate the negative impact of this mycotoxin on piglet performance. Mean feed efficiency in treatment 2 was statistically similar to other treatments, excepted for treatments 1 and 6 *(P<0.01*).

1.2. Use of dietary nitrogen

Nitrogen (N) is an irreplaceable constituent of amino acids that are required for protein synthesis. The quantity of ingested N, absorbed N absorption and retained N in piglets is also a factor influencing growth performance.

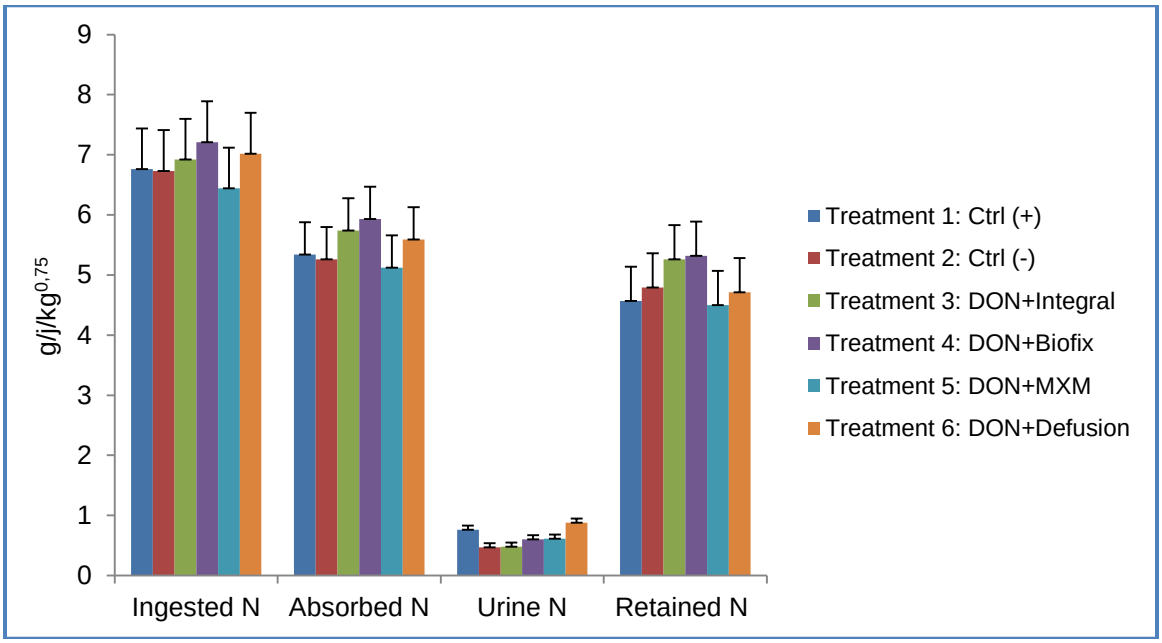

Figure 2. Use of dietary nitrogen of pigs

Each diet contains approximately 4% nitrogen in feed compositions (table 3). The amounts of ingested nitrogen, absorbed nitrogen, urine nitrogen and retained nitrogen of piglets fed with different diets are illustrated in figure 2. No difference was observed for ingested, absorbed and retained nitrogen for different treatments. In contrast, there were significant differences in urine nitrogen; mean of urine excretion in treatment 2 was significantly smaller than in treatments 1 and 6 (*P<0.05*).

1.3. Nitrogen utilization ratio

N digestibility of piglets in treatment 2 was significantly lower than in treatments 3 (*P<0.01*) and 4 (*P<0.03*). However, there were no significant differences between treatment 2 and other treatments. There were significant differences in the ratio of retained N to absorbed N between

treatment 2 and treatments 1 (*P=0.*03) and 6 (*P=0.002*). However for retained N to ingested N there was only a significant difference between treatment 2 and treatment 3 (*P=0.03*).

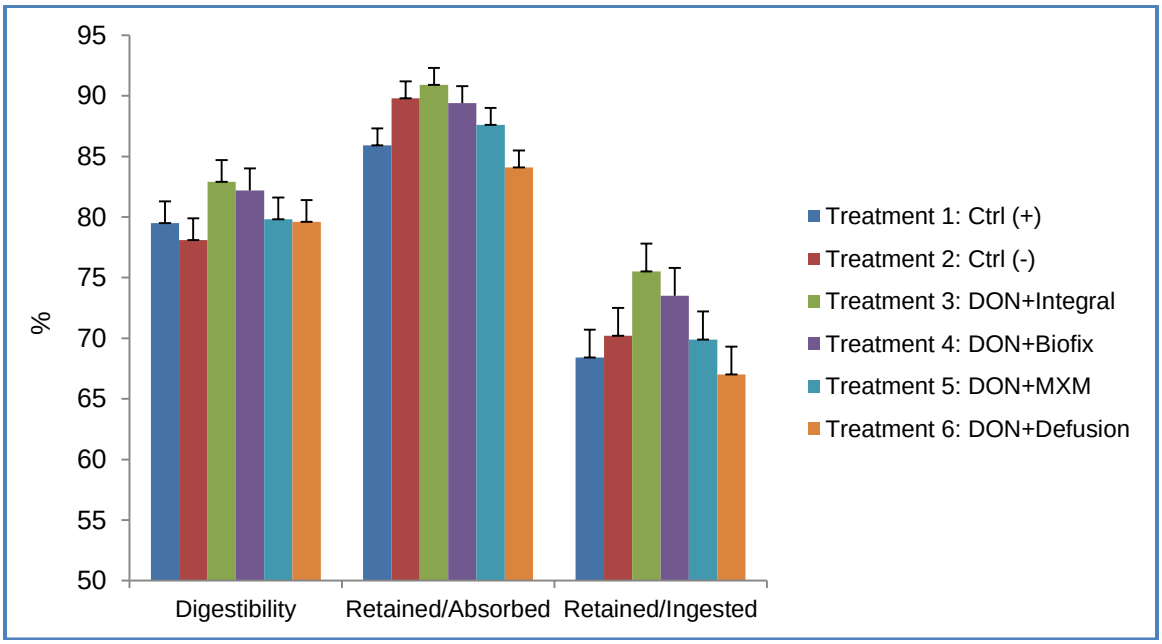

Figure 3. Nitrogen digestibility and nitrogen utilization ratio

1.4. Use of dietary phosphorus

Phosphorus (P) is an essential component of many organic and inorganic compounds in vertebrates such as pigs. Utilization of dietary phosphorus not only depends on the capacity of the piglet's intestinal tract to absorb P, but also on differences in the availability of dietary P between the various feed ingredients.

There were no significant difference between the six treatments for ingested P(*p>0.05*). (figure 4). Absorbed and retained P of piglets on different diets was also measured in parallel with ingested P and P excreted in urine. We only found a significant difference between treatments 2 and 4 for absorbed P (*P=0.04*). No significant differences among treatments were noted for retained P (*P>0.05*). Piglets in treatment 1 eliminated the P in urine to a greater extent than those in treatment 2 (*P=0.0004*). A similar difference was observed between treatments 2 and 6 (*P=0.009*).

1.5. Phosphorus utilization ratio

The P digestibility ranged between 55 and 65%. Piglets in treatment 4 had higher P digestibility than piglets fed treatment 2. Interestingly, the ratio of retained P to absorbed P showed that piglet in treatments 1 and 6 had lower value than piglets fed negative control (*P=0.001*). There were also significant differences between treatments 2 and 1 (*P=0.04*) and between treatments 2 and 6 (*P=0.03*) for ratio retained P to ingested P.

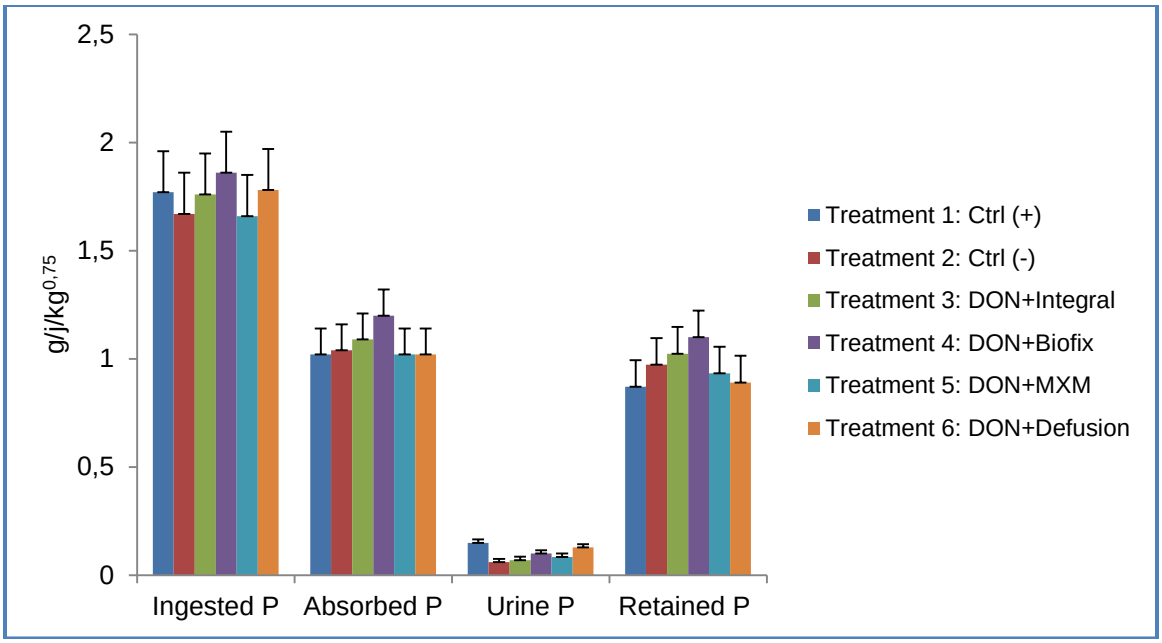

Figure 4. Use of dietary phosphorus

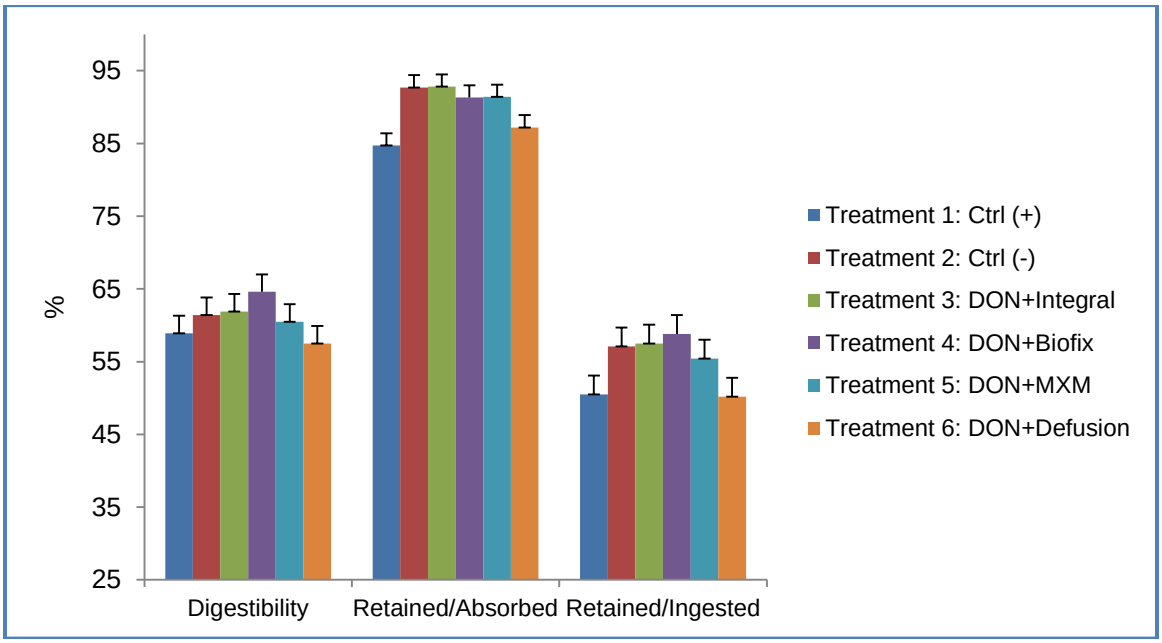

Figure 5. Phosphorus digestibility and phosphorus utilization ratio

1.6. Use of dietary calcium

Piglets in treatment 6 consumed more calcium than piglets in treatment 2 (2g/d/$BW^{0.75}$). Piglets from treatment 2 consumed also lesser Ca than piglets from treatments 4, 6 and 1 (*P<0.05)*. However, statistically significant difference was noted for absorbed Ca between groups of piglets. The amount of Ca excreted in the urine was also measured. Significant differences in Ca excretion were noted between piglets in treatment 2 *vs* those in treatment 6, treatment 1 and

treatment 4 (*P<0.05*). Piglets in treatment 1 retained less Ca than those in treatment 2.. A significant difference was also noted for treatment 2 *vs* treatment 6 (*P=0.02*). No significant difference was found for treatment 2 *vs* treatments 3, 4 and 5.

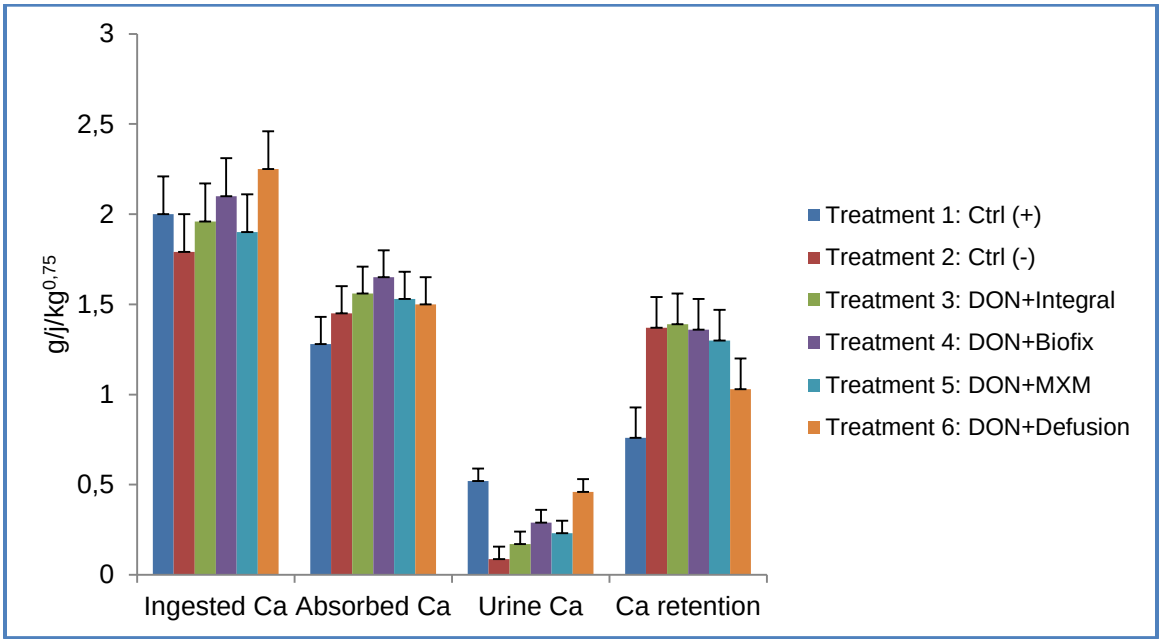

Figure 6. Use of dietary calcium

1.7. Calcium utilization ratio

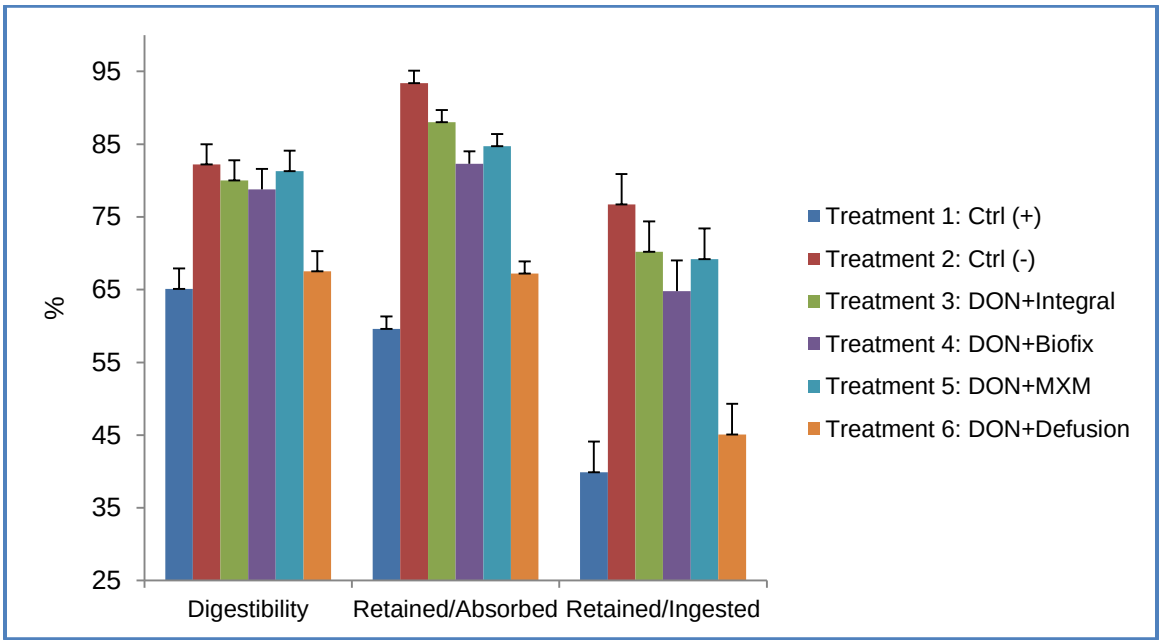

Figure 7. Calcium digestibility and calcium utilization ratio

The amount of Ca being digested by the piglets is within in the range of 65% to 85%. Piglets in treatment 2 was better Ca digestibility than piglets from treatment 1 and 6 (P<0.05). The figure 7 showed that piglets in treatment 2 had higher ratio retained Ca to absorbed Ca than treatments 1 and 6 ($P<0.05$). There were also significant differences between treatment 2 and treatments 1, 4 and 6 ($p<0.05$) for ratio retained Ca to ingested Ca.

2. Effects of DON contaminated food and feed additives on nutrient digestibility

2.1. Nutrient digestibility

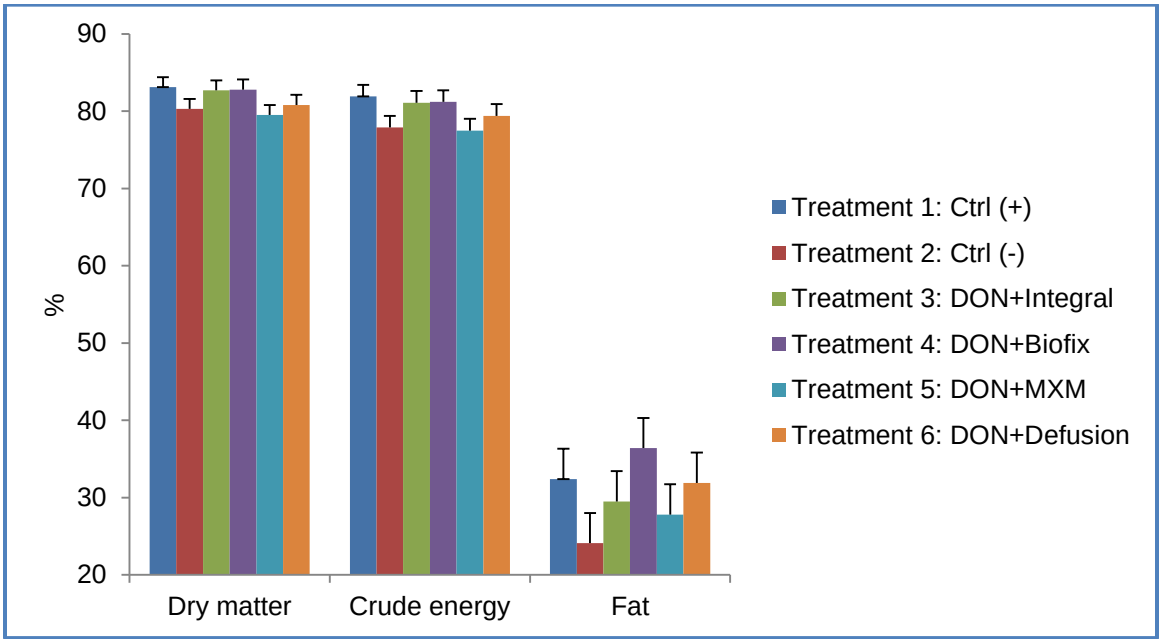

Figure 8. Nutrient digestibility

We could only find a significant difference between treatment 2 and treatment 1 ($P<0.05$). The differences between treatment 2 *vs* treatment 3 and treatment 4 tended to be significant ($P=0.06$ and $P=0.05$, respectively). There were significant differences between treatment 2 and treatment 1 ($P<0.05$) for crude energy digestibility as well as between treatment 2 *vs* treatments 3 and 4 ($P<0.05$). We also evaluated fat digestibility in piglets fed different experimental diets. Those receiving treatment 4 had higher fat digestibility than piglets in treatment 2 ($P<0.05$). Fat digestibility was lower in treatment 2 than in treatment 1 and treatment 6 ($P<0.05$).

2.2. Intestinal crypts and villi

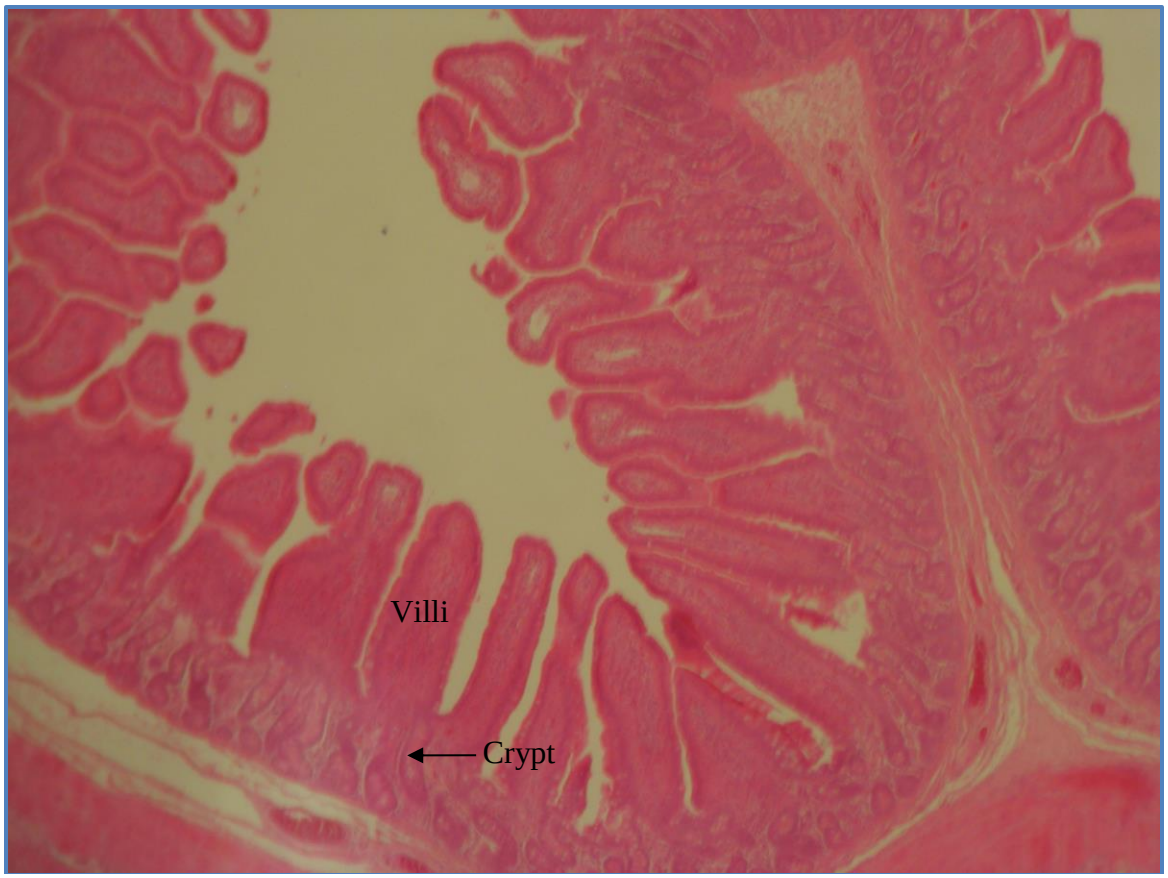

Figure 9. Intestinal crypts and villi of pig's jejunum

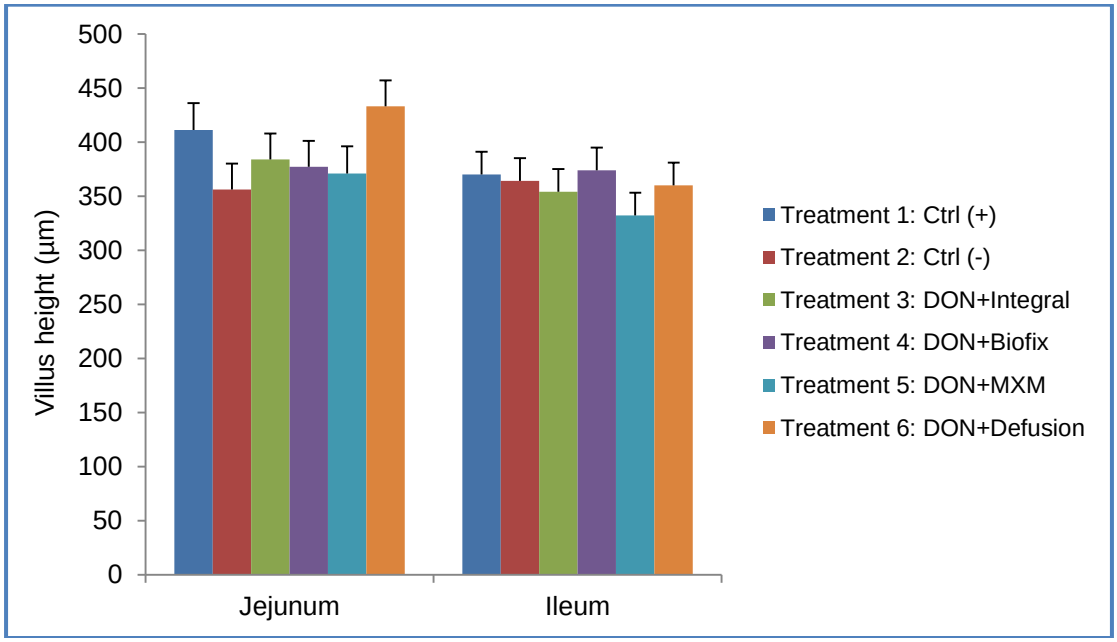

Figure 10. Height of intestinal crypts and villi

No significant difference was found between treatments (*P>0.05*) for the ileum villi height as well as for ileum crypt depth. A significant difference was observed for jejunum villi height between piglets in treatments 2 and 6 (*P=0.007*) and between treatment 1 and 2 (*P<0.05*). No significant difference can be found for jejunum crypt depth (*P>0.05*).

Table 1: Urinary recovery (% of administered dose) of lactulose and mannitol, and urinary lactulose:mannitol ratios of weaning piglets fed diets contaminated with DON and supplemented with different anti-mycotoxin additives.

	1-Ctrl (+)	2-Ctrl (-) (DON)	3-DON +Integral	4-DON +Biofix	5-DON +MXM	6-DON +Defusion	SEM
Mannitol recovery[a], %	18.1	8.7	8.7	11.1	13.7	7.7	2.9
Lactulose recovery[abc], %	1.78	0.64	1.11	0.78	1.64	1.70	0.61
Lactoluse_Mannitol ratio	0.097	0.142	0.086	0.072	0.137	0.216	0.63

[a]Ctrl (+) vs Ctrl (-) DON, P<0.05
[b]Ctrl (-) DON vs DON+MXM, P<0.07
[c]Ctrl (-) DON vs DON+Defusion, P<0.07

No significant difference was found between treatments (*P>0.05*) for lactulose_mannitol ratio. A significant difference was observed for mannitol and lactulose recoveries between treatment 1 and 2 (*P<0.05*) and tendencies were noted between treatment 2 and 5 and 6 (*P<0.07)*.

3. Development of a rapid indicator of exposure to DON allowing early detection of mycotoxicosis in piglets

3.1. DON and DOM-1 concentration in serum

DON and DOM-1 concentrations were determined in the serum at the mid-point and at the end of the experimental periods (DOM-1 is a DON metabolite). Significant differences in serum DON concentrations of piglets receiving treatment 2 *vs* those receiving treatment 1 (*P<0.0001*) and treatment 6 (*p=0.007*) were noted. However, no significant difference for DOM-1 in serum can be detected among treatments.

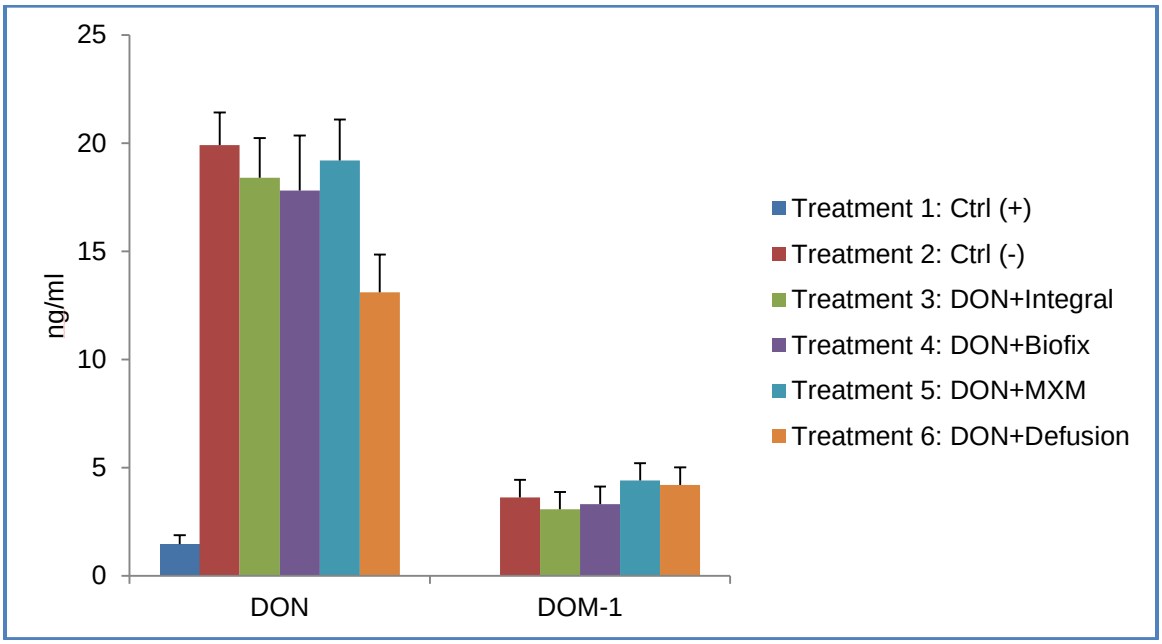

Figure 11. DON and DOM-1 concentration in serum

3.2. Intestinal enzyme activities: disaccharidases and aminopeptidase N

3.2.1. Disaccharidases

In the present study, intestinal disaccharidase activities (lactase and sucrase) of jejunum and ileum mucosa were analyzed (figures 12 and 13). The activity of sucrase in jejunum intestine varied little among treatment groups. No significant differences in sucrase activity were found in jejunum intestine (*p>0.05*). Activity of lactase in jejunum intestine did not vary statistically among treatments (*p>0.05*). The same lack of difference was noted in the ileum intestine (*p>0.05*).

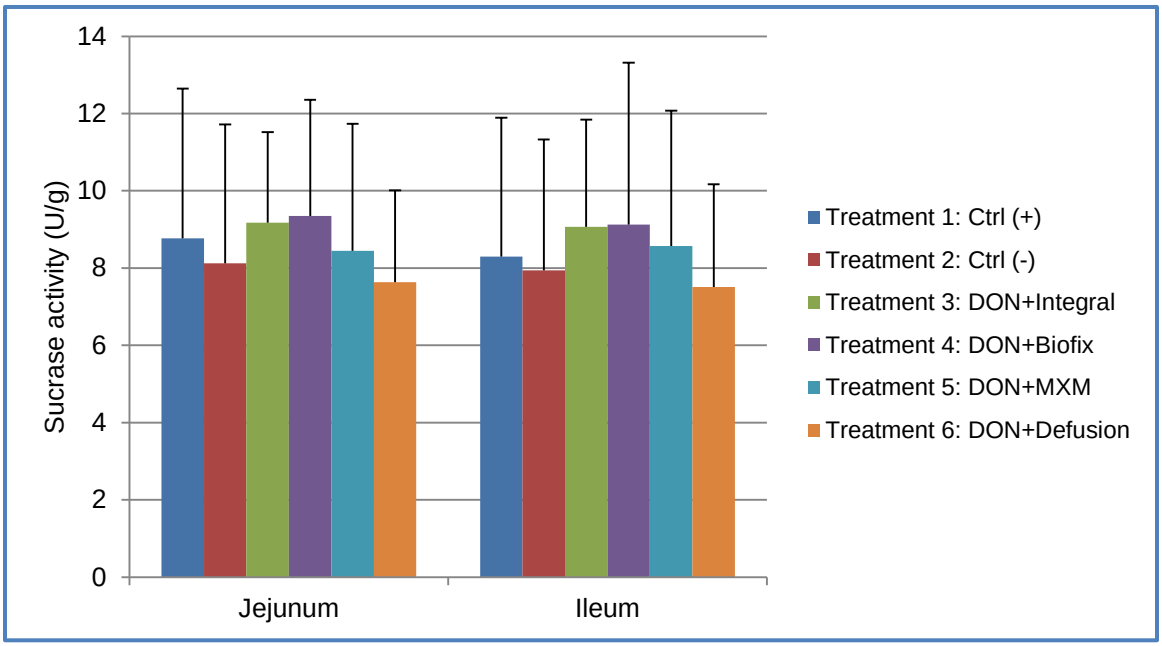

Figure 12. Sucrase activity of jejunum and ileum intestine

The activity of sucrase was also evaluated in ileum intestine. No significant difference in sucrase activity were found in ileum intestine (*p>0.05*).

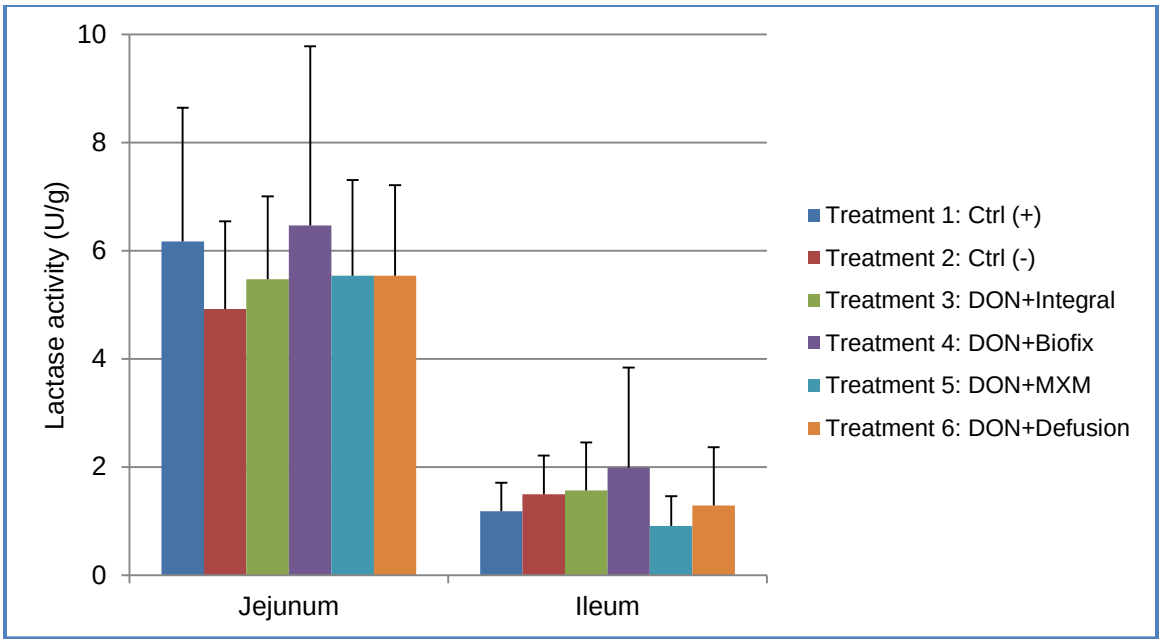

Figure 13. Lactase activity of jejunum and ileum intestine

3.2.2. Aminopeptidase N

In the small intestine, aminopeptidase N plays a role in the final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases. Aminopeptidase N activity in this study is presented in figure 14. The value of aminopeptidase N activity in the jejunum segments did not vary statistically among treatments ($p>0.05$).

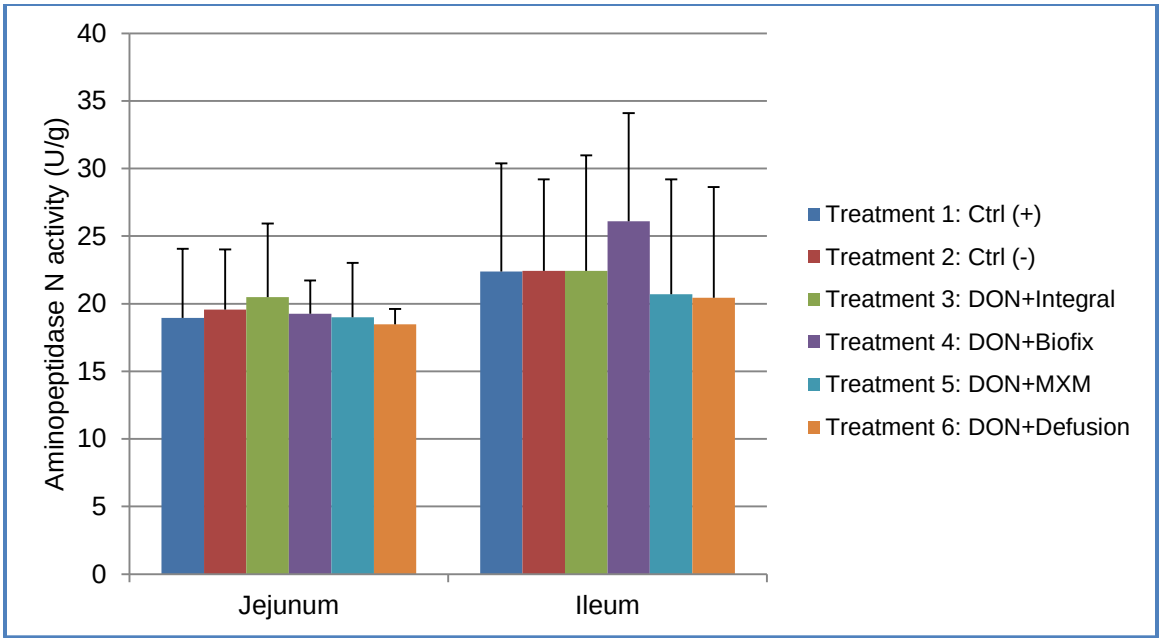

Figure 14. Aminopeptidase N activity of jejunum and ileum intestine

6 Lessons learned and best practices

Please comment on the unanticipated outcomes/benefits as well as the strengths, opportunities, and challenges of the Project. Describe any follow-up activities, along with their potential outcomes in terms of commercialization and/or transfer to the end-user.

Activity 2. In vitro, DON concentrations of 560 ng/ml and higher are detrimental to cell line such MARC-145 and primary porcine alveolar macrophage cells (PAM). PRRSV strains used in this study induced a rapid cell death. However, exposure to sub-toxic concentrations of DON reduced cell mortality triggered by PRRSV, in a dose dependant manner. Sub-toxic DON doses could inhibit PRRSV replication which may explain decreased cell mortality caused by DON in infected cells. Very likely, the early activation of pro-inflammatory genes and apoptosis following DON exposure appear to be detrimental to PRRSV survival in MARC-145 and PAM cells.
NPTr cells are also very sensitive to the toxic effect of DON as previously expressed by cell viability and mortality. The effect of DON on PCV2 infected NPTR cells was dose and genotype dependant; PCV2b replication could be enhanced by the addition of DON at sub-toxic concentrations while those of PCV2a would be rather reduced. High concentrations of DON However seem to strongly reduce viral replication of PCV2 regardless of genotypes.

In vivo studies showed that ingestion of diets highly contaminated with DON greatly increases the effect of PRRSV infection on weight loss, lung lesions and mortality, without increasing significantly PRRSV replication.
It is difficulty to reproduce severe clinical expression of PCV associated disease through experimental infections with PCV2 alone. No clear potentiating effect of DON mycotoxin on PCV2 disease was observed in this study, even if the replication of the virus was slightly increased in the group ingesting feed containing 1.5 ppm/kg of DON. However, growth performances tend to demonstrate a beneficial effect of DON rather than the development of clinical signs.
In both in vivo studies, DON impacted PRRRV and PCV2 antibody responses indicating that this mycotoxin could undermine the efficacy of live attenuated vaccine against these viruses in pig.

Activity 3. In this study, the feed additive Defusion proved to be a more effective mycotoxin inhibitor than the three other additives tested, as shown by a higher average daily gain and a higher average daily feed intake during the 14 days of the experimental period. The effects of the other mycotoxin inhibitors on growth performance, nutrient digestibility and DON concentration in blood were similar to that in the negative control.

Defusion also demonstrated its effectiveness against DON; however we did not test the various supplements against mycotoxins other than DON such as zearalenone, T-2 toxin or aflatoxin. The cereals used in our diets were purposely contaminated with DON, but they may also have been contaminated by other mycotoxins against which the different supplements could have been effective to varying degrees. Thus, we cannot assess how the four mycotoxin inhibitors used in this study would have responded to contamination with different mycotoxins or combinations of mycotoxins.

This study sets the stage for more elaborate trials that would be necessary in order to either validate or refute our findings. The study provides a guideline for the methodology to be followed and a basis upon which to evaluate the various costs (material, labour, animals, etc) of such trials. The outcome of these trials may lead to enhanced knowledge concerning the effect of mycotoxin inhibitors on growth and other performance parameters in piglets.

The ensuing information may lead to recommendations on the most appropriate ways to use the various commercial mycotoxin inhibitors individually or in combination. An additional benefit resulting from another aspect of this study will materialize once the rapid test for detection of mycotoxins in serum and urine is elaborated.

7 Benefits to Canada

Please state the benefits to Canada of work carried out in your Project. Include mention of how the project addressed issues of high priority for the swine sector and Canada, how the project has yielded benefits (e.g. economic, environmental and/or social) to the swine sector and Canada, how the Project has strengthened links between researchers and the agricultural, agri-food and agri-based products sector, and identify any potential long-term impacts that might be realized after March 31, 2013 that will benefit the Canadian swine and agricultural sectors.

Activity 2. In this project, effects of deoxynivalenol contaminated feed on susceptibility to viral infections PCV and SRRPV and on immune response in piglet were evaluated. Circovirus and SRRPV are major viruses in Canada causing important viral diseases with significant production and economic losses, reproductive disorders and respiratory problems. On the other hand, deoxynivalenol (DON, vomitoxin), a trichothecene, is prevalent worldwide in crops used for food and feed production, including in Canada. Although DON is one of the least acutely toxic trichothecenes, it is an important feed safety issue because it is a very common contaminant of grain grains intended for pig feeding in Canada. High concentrations of DON in pig diet enhance animal susceptibility to viral infections particularly to PCV and SRRPV. Controlling feed quality with lower DON concentrations should help animals infected with these viruses the exacerbation of their health conditions.

Activity 3. Reducing the risk of deoxynivalenol (DON) intoxication in raising piglets is a relevant topic in animal nutrition. Indeed, high humidity, typical of Canada, favour the development of Fusarium in cereals. Discovering mycotoxin inhibitor products such as Defusion, which can mitigate the effects of DON in pig rations on growth performance, supplement is a good opportunity to use contaminated grains less expensive for pork producers for swine producers.

8 Major impact on budget

Please state the impact of changes over the term of the Project on the most recently approved CSRDC budget.

Activity 2. The budget dedicated to this project allows good quality results and a master degree student training and open the door for many collaborations between all the researchers implicated in the project.

Activity 3
2010-2011
The budget and timelines have been met to date

2011-2012
Salary: Planned: $ 17,500 Spent: $ 18,000

The annual amount for the graduate student, planned to $ 15,000, increased to $ 18,000 since a PhD student realized this project. The amount allocated ($ 2,500) for the professional research CDPQ will be paid in 2012-2013.

Supplies: Planned: 29,000$, Spent: 10,387.23

Service contracts: Planned $ 0.00 Spent: $ 16,965.70

It should be added to $10,387.70, $ 16,965.0- from to service contract. In fact, this amount includes the costs to use equipments and supplies charged by Laval University (Laboratory analysis).

2012-2013

See the statement of expenditure of Laval University

9 Performance management report

For this section, please use the accompanying Excel workbook titled, 'CSRDC_Performance Management Report'. Worksheets include: Innovation, Technology Transfer, Capacity Building, Success Stories, Media, Recognition, Publication, and Challenges & Concerns. Detailed instructions for completing the worksheets are provided in the workbook. Please note that a Success Story could present a significant research result, successful technology transfer, potential for pre-commercialization, and anticipated impact on the sector and Canada.

IMPORTANT: Please include copies of all Communications Materials and Products that acknowledge the role of the CSRDC along with the government of Canada in keeping with '10 Public Announcements' of your CSRDC Contribution Agreement.

Signature Page

The signature below confirms that the information in this Final Report for Efficacy of feed additives to mitigate the negative impacts of mycotoxin contaminated feed on performance and health of piglets [1014] for the project end date of December 31, 2012 is complete and accurate.

Authorized person	Date

Please send an electronic copy of this completed document as well as a signed original copy to:

ATTN: Abida Ouyed, M. Sc.
Canadian Swine Research and Development Cluster (CSRDC)
Place de la Cité, tour Belle Cour
2590, boul. Laurier, suite 450
Québec (QC) G1V 4M6
aouyed@swineinnovationporc.ca

And, when sending an electronic copy regarding CSRDC projects #1011, #1012 or #1016, please include a 'cc' to:

Susan Joyal, M.Sc.
CSRDC Research Coordinator – Western Canada
sjoyal@cdpqinc.qc.ca