Effects of deoxynivalenol exposure time and contamination levels on rainbow trout

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Keywords:	Mycotoxins, Oncorhynchus mykiss, pathogen susceptibility, hepatocytes hyalinization

Effects of deoxynivalenol exposure time and contamination levels on rainbow trout

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Running title: Effects of deoxynivalenol on rainbow trout

Abstract

The trend towards using plant-based ingredients in aquafeeds is set to intensify; however, mycotoxin contamination might be a challenge. Two diets, with deoxynivalenol (DON) levels of 1,166 µg kg^{-1} (1.1 DON) and 2,745 µg kg^{-1} (2.7 DON), were prepared for short-term DON-exposure (50 days). A third diet with a low DON level of 367 µg kg^{-1} (0.3 DON) was prepared for long-term DON-exposure (168 days). Ingestion of DON by trout during both short-term/high-dosage exposure (50 days; 1,166 µg kg^{-1} and 2,700 µg kg^{-1} DON) and long-term/low-dosage exposure (168 days; 367 µg kg^{-1} DON) impacted growth performance and, to a lesser extent, liver enzyme parameters (2.7 DON). Histopathology showed mild to moderate changes in the liver but not in the other

sampled tissues (intestine and kidney). Despite these effects, short-term exposure of rainbow trout to high doses of DON did not result in increased susceptibility to *Yersinia ruckeri*. In both the short- and long-term studies, the effects of DON showed a high inter-individual variability. The present study confirms that sub-clinical levels of mycotoxins affect rainbow trout. The effects of such low mycotoxin levels could be masked by other production challenges while still negatively affecting productivity.

Keywords: Mycotoxins, *Oncorhynchus mykiss*, pathogen susceptibility, hepatocytes hyalinization

Introduction

In aquaculture, the trend to replace expensive animal-derived proteins, such as fishmeal, with more economical and sustainable plant protein sources has increased the probability of mycotoxin contamination in aquaculture feeds. According to Tacon *et al.* (2011), plant-based ingredients already represent the major dietary protein source used in feeds for lower trophic level fish species, such as tilapia, carp and catfish. These ingredients also account for the second major source of dietary protein and lipids after fishmeal and fish oil in the feed of shrimp and high trophic level fish species. Various plant sources have been used for salmonids but at lower inclusion levels than feed destined for lower trophic species. In most aquaculture species, plant protein choice and selection are based on a combination of local market availability, cost and the nutritional profile (including anti-nutrient content and level) of the plant meal in question (Gatlin *et al.,* 2007; Davis and Sookying, 2009; Krogdahl *et al.,* 2010).

The mycotoxin contamination of finished feeds and raw materials used in aquaculture as well as the negative effects of mycotoxins on aquatic species, particularly rainbow trout (*Oncorhynchus mykiss*), has been highlighted in recent publications (Hooft *et al.*, 2011; Ryerse *et al.*, 2015; Tola *et al.*, 2015; Gonçalves *et al.*, 2018; Hooft and Bureau, 2017). However, mycotoxin contamination is not generally assessed in commercial aquafeeds or plant meals used to manufacture these feeds. Consequently, we do not have accurate estimates of the mycotoxin contamination levels in these commodities.

Few studies are currently available concerning mycotoxin occurrence in aquaculture plant meals and finished feeds. Gonçalves *et al.* (2016) reported that deoxynivalenol (DON) was present in 68% of analyzed samples (shrimp and fish, sampled in Asia and Europe in 2014) at average contamination levels of 162 µg kg^{-1} and maximum levels of 413 µg kg^{-1}. More recently, Gonçalves *et al.* (2018) observed that contamination patterns for shrimp and fish feeds were slightly different, which likely reflects the type of commodity used for the different species. The authors observed that shrimp feeds were generally contaminated with low levels of DON, with the exception of some diets (contamination ranging from 329 µg kg^{-1} to 2,287 µg kg^{-1} of DON). In the case of fish feeds, samples were contaminated mainly by DON, up to a maximum level of 396 µg kg^{-1}, and were co-contaminated with other mycotoxins.

Trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis, interfering with the initiation, elongation, and termination stages of this process (Kumar *et al.*, 2013). Knowledge of the effects of DON on aquatic species has increased recently (Hooft *et al.*, 2011; Matejova *et al.*, 2015; Ryerse *et al.*, 2015; Tola *et al.*, 2015; Hooft and Bureau, 2017; Gonçalves *et al.,* 2018), and studies on rainbow trout suggest that DON has a detrimental effect on feed intake, weight gain and feed efficiency (Hooft *et al.*, 2011; Ryerse *et al.*, 2015). Curiously, no effect has been detected on the immune status of animals fed with DON (Matejova *et al.*, 2015; Matejova *et al.*, 2017; Ryerse *et al.*, 2015).

In general, the effects of mycotoxicoses vary greatly depending on a variety of factors, including nutritional and health status prior to exposure, dose and duration of exposure, age, species and infection route. In addition, the lack of reliable clinical signs or parameters (including biomarkers) to correctly diagnose the ingestion of DON by aquatic species makes mycotoxin risk management in aquaculture very challenging.

The aim of the present study was to evaluate the effect of DON on rainbow trout under two different scenarios: first, the effect of short-term feeding of high levels of DON (50 days; 1,166 µg kg^{-1} DON and 2,745 µg kg^{-1} DON), and second, the effects of long-term feeding of low levels of DON (168 days; 367 µg kg^{-1} DON). Moreover, we aimed to investigate the manifestation of clinical signs due to the ingestion of DON by inspecting several organs and tissues normally affected by the consumption of mycotoxins.

Materials and methods

Experimental diets

The experimental diets were formulated to be isoenergetic (22.20 kJ g^{-1} dry matter (DM), isoproteic (52.20% DM) and isolipidic (17.90% DM) (Table 1)). All diets were formulated with the same ingredients. Marine-derived ingredients (fishmeal and fish oil) represented 22.45% DM of the diet, whereas plant raw materials represented 59.70% DM of the diet. All ingredients were finely ground (hammer mill, 0.8-mm sieve), mixed, and then extruded (twin screw extruder, 2.0-mm pellet size, SPAROS, Portugal).

The ingredients used to formulate the diets were subjected to Liquid chromatography-tandem mass spectrometry, HPLC-MS/MS-based multi-mycotoxin analysis (University of Natural Resources and Life Sciences, Center for Analytical Chemistry Department IFA, Austria), as described by Streit *et al*. (2013). The method covered major type A and B trichothecenes, zearalenone, fumonisins, aflatoxins and ochratoxins. For the purpose of data analysis, non-detect levels were based on the limits of detection (LOD) of the method used for analysis. The

detected concentrations of major mycotoxins and of a selection of other fungal metabolites are listed in Table 2.

Diets with three different levels of DON were prepared by adding DON (Romer Labs Diagnostic GmbH, Austria) to the feed during diet ingredient mixing. Two diets, with DON levels of 1,166 µg kg^{-1} (1.1 DON) and 2,745 µg kg^{-1} (2.7 DON), were prepared for short-term DON exposure (50 days). A third diet with a low DON level of 367 µg kg^{-1} (0.3 DON) was prepared for long-term DON exposure (168 days). All diets were dried at 45 C for 12 hours after the addition of DON and were stored at 4 C until use.

Contamination levels were chosen taking into account previous literature on the effect of DON on rainbow trout (Hooft, Elmor *et al*., 2011; Matejova *et al*., 2014; Matejova, Vicenova *et al*., 2015; Ryerse, Hooft *et al*., 2015) as well as the reported DON levels in worldwide finished feed samples (Gonçalves *et al*., 2016, 2017, 2018; Greco *et al*., 2015; Barbosa *et al*., 2013). The long-term exposure to DON attempts to mimic the most recently reported levels of DON in finished feeds (Gonçalves *et al*., 2018, average of 82.87 µg kg^{-1} and maximum of 396 µg kg^{-1}). However, the authors are aware that reports of mycotoxin occurrence in European aquaculture finished feeds are still very limited, and levels reported may vary annually (e.g., average DON contamination of 160.86 µg kg^{-1} in 2014, of 165.61 µg kg^{-1} in 2015, and of 87.87 µg kg^{-1} in 2016; Gonçalves *et al*., 2016, 2017 and 2018). Generally, Asian aquafeed samples present higher DON levels compared with European aquafeed samples.

Fish and experimental conditions

This study was approved by the institutional ethics committee and the national authority according to §26 of Law for Animal Experiments, Tierversuchsgesetz 2016—TVG 2012 under No. BMWFW- 68.205/0135-WF/V/3b/2014. Rainbow trout (*Oncorhynchus mykiss*) originating from a farm with no prior history of *Yersiniosis* was used in both experiments. On arrival, the kidneys of ten fish were sampled, and their infection-free status was confirmed by

culture-based analysis and polymerase chain reaction (PCR)-based analysis using *Yersinia ruckeri* specific primers (del Cerro *et al.*, 2002).

Short-term exposure to DON

For the experiment with short-term exposure to DON, 180 fish (14.10 ± 0.05 g) were randomly allocated to three feeding groups in quadruplicate and given either standard feed (control, CTRL), feed contaminated with 1,166 µg kg^{-1} DON (1.1 DON) or feed contaminated with 2,745 µg kg^{-1} DON (2.7 DON). Each aquarium of 85 L was supplied by a flow-through system with a temperature of 15.47 ± 0.14 C, oxygen concentration of 8.73 ± 0.12 mg L^{-1}, and pH of 7.53 ± 0.04, with 0.0 ± 0.0 mg L^{-1} total ammonia nitrogen, nitrites and nitrates. The fish were hand-fed the prepared diets (CTRL, 1.1 DON or 2.7 DON) three times per day near satiety for 50 days prior to performing the *Y. ruckeri* challenge.

Long-term exposure to DON

For the long-term exposure experiment, 120 fish weighing 89 ± 8 g were randomly allocated and distributed among eight tanks, each with a volume of 1 m^3, supplied by a flow-through system with a water temperature of 18.6 ± 1.0 C, oxygen concentration of 8.56 ± 0.26 mg L^{-1} and pH of 7.35 ± 0.35. Each tank contained 15 fish that were fed restrictively (2.5% of the average body mass) with either control feed (CTRL, 4 tanks) or the control feed supplemented with 367 µg kg^{-1} DON (0.3 DON, 4 tanks) for 168 days. The same quantity of feed (2.5% of the average body mass) was distributed in each tank by hand feeding and was adjusted after intermediate weighing periods (at 37, 62, 92 and 125 days). Five fish per replicate tank were subjected to moderate anesthesia (tricaine methanesulfonate (MS222) (Sigma-Aldrich Co., LLC, Bellfonte, USA) at a dose of 0.7 g L^{-1}, and a blood sample was collected by puncture of the caudal vein with a heparinized syringe at the beginning of the trial and at 62 and 125 days. Part of the blood sample was used for the determination of hematocrit, which was determined for five fish per treatment. Blood was transferred into hematocrit capillary tubes

(Hirschmann), the tubes were then centrifuged at 13,000 RPM for 5 minutes (Hettich Haematokrit 200), and the percentage of red blood cells to sera was measured. The remaining part of the blood sample was centrifuged at 1,590 x g for ten minutes, after which the plasma (i.e., the supernatant fraction) was transferred to Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80 C until subsequent analysis of total protein. Total protein was determined by the Bradford method (Bradford 1976) using bovine serum albumin as the standard. All measurements were performed in a Synergy HT multi-mode microplate reader (BIOTEK, Vermont, USA).

Growth performance

All fish, in both the short- and long-term exposure experiments, were weighed to determine the initial individual body weight at the start of the experiments. In the short-term exposure study, the fish were weighed individually at the end of the 50-day period, and their total length was measured and recorded. Feed intake was recorded daily. In the long-term exposure study, the fish were weighed individually after 37, 62, 92,125 and 168 days.

The following calculations were made in both experiments.

The thermal-unit growth coefficient (TGC) was expressed as the growth rate and was calculated for each aquarium as [100 × (FBW 1/3 – IBW 1/3) / Σ (Temp (°C) × number of days)], where FBW = final body weight (g fish $^{-1}$) and IBW = initial body weight (g fish $^{-1}$).

The feed conversion ratio (FCR) was calculated as crude feed intake/weight gain, where FI = total dry feed/number of fish.

The protein efficiency ratio (PER) was calculated as weight gain (g)/protein intake (g).

The specific growth rate (SGR) was calculated as [(ln final weight − ln initial weight)/time in days] × 100).

Fulton's condition factor, K, was also used to measure individual fish health: $K = 100(BW/L^3)$, where BW is the whole body wet weight (g) and L is the length (cm). A factor of 100 was used to transform K to approximate a value of one.

Liver enzymes

In the short-term/high DON exposure experiment, five fish from each aquarium were sampled at the end of the experiment (50 days) for analysis of liver enzymes in blood. In the long-term/low DON exposure study, five fish from each aquarium were sampled on day 62 and on day 125. The fish were anesthetized by immersion in tricaine methanesulfonate (MS222) (Sigma-Aldrich Co., LLC, Bellfonte, USA) at a dose of 0.7 g L^{-1} prior to blood collection. Blood samples were analyzed to measure the activities of lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate aminotransferase (AST) using a Spotchem EZ SP-4430 reader and Spotchem II GPT/ALT, Spotchem II LDH and Spotchem II GOT/AST kits (all products from Arkay, Amstelveen, Netherlands).

Histological examination

For the short-term/high DON exposure study, organs were sampled from 10 fish prior to the *Y. ruckeri* challenge and at the time of termination. The intestine, spleen, liver and kidneys (head and trunk kidney) of the fish were removed and fixed in 10% buffered formalin for 48 to 72 hours. The samples were embedded overnight in paraffin using a HistoMaster (Formafix, Düsseldorf, Germany). Sections (3 - 4 µm thick) were cut from each paraffin block and were left to dry overnight at 37 C before being stained with hematoxylin and eosin. The slides were evaluated under a light microscope (Nikon Eclipse E400, Feasterville, Pennsylvania). The following were examined: intestine (number of mucous cells in mucosa), liver (hepatocyte vacuolation, hepatocyte hyalinization, single cell necrosis, number of pigmented macrophage centers, perivascular and peribiliary inflammation), and kidney

(number of pigmented macrophage centers). To evaluate the number of cells, three high-power fields (HPF) were counted per slide.

Bacterial preparation

As a pre-trial to the challenge test, five groups of ten fish each were challenged by immersion with *Y. ruckeri* isolate 7959/11 to determine the appropriate infectious dose. *Y. ruckeri* isolate A7959/11 is a clinical isolate that originated from an outbreak at an Austrian trout farm in 2011. This isolate was kept at -80 C on beads until three days prior to the start of the experiment. It was then inoculated on a blood agar plate and incubated at 22 C. After 48 hours, a single colony was inoculated into 7.5 ml of BHI broth and was incubated in a shaking incubator at 20 C with rotation at 150 rpm. After 10 hours, the cultures were evaluated by eye, and 2.5 ml was sampled from one culture and used to inoculate a 1.5-L BHI broth. This broth was then incubated for approximately 12 hours at 20 C with shaking at 150 rpm.

Infection trial in the short-term exposure study

After 50 days, each feeding group of the short-term/high DON exposure study was further divided into two groups: two of the aquaria were infected with *Y. ruckeri* while fish in the two other aquaria were mock-infected with un-inoculated broth. In total, 90 fish were infected and 90 were mock-infected. The infection procedure was adapted from that described for *Aeromonas salmonicida* (Menanteau-Ledouble *et al.*, 2017). Briefly, bacteria were grown overnight in 1.5 L of BHI broth and their concentration was determined by measuring the optical density at a wavelength of 600 nm (OD600) per ml. Water circulation in the aquarium was interrupted, and the water volume was lowered to 50 L. The bacterial culture (2 ml) was added to each of the aquaria, yielding a final concentration of $2x10^4$ CFU mL^{-1}. The fish remained in the solution for two hours, after which the water was progressively returned to its normal level and the circulation was reopened. The fish were monitored at least twice daily.

Mortalities were recorded, and dead and moribund fish were immediately removed from the tanks. Moribund fish were euthanatized by prolonged immersion in a solution of 1 g L^{-1} of MS-222, and the kidney of the fish was sampled for microbial re-isolation of the pathogen on an agar plate. The colonies growing on these plates were examined and confirmed to be *Y. ruckeri* based on their morphologies. Furthermore, one in five isolates was selected; its genomic DNA was isolated using a Qiagen DNeasy kit, and PCR was performed using *Y. ruckeri* specific primers (del Cerro *et al.*, 2002). The surviving fish overcame the infection 17 days post-infection, at which point the challenge was terminated. All remaining fish were euthanatized by prolonged immersion in a solution of tricaine methanesulfonate (MS222; 1 g L^{-1} of water), weighed, measured and examined for gross clinical signs of enteric red mouth syndrome (oral congestion, hemorrhages or petechia, exophthalmia and ocular hemorrhages, ascites in the abdominal cavity, enlarged spleens and hemorrhages or petechia in the internal organs, bloody intestines or adipose tissues).

Clinical signs

During both experiments, gross clinical signs were assessed by visual examination of the fish at the time of termination. Lesions (hemorrhages and ulcerations) on the skin were recorded, as were any obvious abnormalities such as a protruding anal papilla. The state of the gills was recorded as well as the presence of anemia, hemorrhages or necrosis.

The fish were examined internally for any abnormalities. In particular, record was made of congestions, petechia or hemorrhages of the internal organs. The color of the liver and the size of the spleen were assessed, as was the general health of the intestine (in particular, the presence of congestion, hemorrhage or intussusception was determined).

Statistical analysis

All parameters such as the final weight, SGR, PER, FI, FCR, condition factor (CF), TGC, LDH, ALT and AST were subjected to analysis of variance in SPSS 21 for Windows (IBM Corp., Armonk, NY, USA). One-way ANOVA was performed, and differences between the means were tested by Tukey's multiple range test. The Shapiro-Wilk test was used to analyze the normality, and homogeneity of variances was tested using Levene's test. Data analyzed did not violate the assumption of equal variances and showed a normal distribution. All parameters expressed as percentages were subjected to arcsin square root transformation. Additionally, one-way ANOVA was performed to analyze the histological differences in the intestine (number of mucous cells in mucosa) and liver (single cell necrosis, number of pigmented macrophage centers, perivascular and peribiliary inflammation) between the DON dietary treatments and controls.

Following the challenge, survival curves were constructed for each treatment, and Kaplan-Meier and odds ratio analyses were performed using SPSS v.20 (IBM) and MedCalc (Microsoft).

The level of significance was set at $p < 0.05$, and the results are presented as the mean ± SD (standard deviation of the mean).

Results

Experimental diets

The four experimental diets were formulated to be isoenergetic (22.20 kJ g^{-1} DM), isoproteic (52.20% DM) and isolipidic (17.90% DM) and to meet all the nutrient requirements for the species examined in the study. There was no significant difference ($p > 0.05$) between treatments regarding the nutritional composition of the experimental diets. Analysis of the feed to confirm mycotoxin levels showed DON contamination was successfully achieved, although observed levels were slightly lower than intended (Table 2). Other metabolites/toxins were found in the basal diet (common to all experimental groups) due to

natural contamination of the plant raw materials used to formulate the diet (Table 2). Generally, these metabolites/toxins, produced mainly by *Fusarium* and *Aspergillus,* were at levels below 100 µg kg^{-1}. Regarding the *Penicillium* toxins, brevianamide F and rugulusovin were found at levels of 194 and 244 µg kg^{-1}, respectively. Fungal and bacterial metabolites were also detected in the experimental diets, namely, cyclo (L-Pro-L-Val) and cyclo (L-Pro-L-Tyr) at relatively high concentrations (1,631 and 2,004 µg kg^{-1}, respectively).

Growth performance

Short-term DON exposure

The results showed that rainbow trout was sensitive to the DON levels tested (Table 3). The presence of 2,700 µg kg^{-1} DON in the diet led to a significant decrease ($p < 0.001$) in FI. The same treatment (2.7 DON) also resulted in a significant decrease in the final weight (79.91 ± 16.54 g; $p < 0.001$), SGR (2.20 ± 0.09% day^{-1}; $p < 0.001$), TGC (0.094 ± 0.005; $p < 0.001$) and CF (1.39 ± 0.12; $p < 0.033$) compared to the controls (final weight = 101.36 ± 19.8 g; SGR = 2.52 ± 0.07% day^{-1}; TGC = 0.113 ± 0.005 and CF = 1.42 ± 0.12). Observations of the feeding behavior of the DON-fed groups confirmed that the fish initially accepted the feed, and a reduction in FI was progressively established. We therefore assumed that the lower FI in the DON-fed groups compared to the control group was probably not due to the unfavorable organoleptic properties of DON-contaminated feed.

Long-term DON exposure

In the long-term exposure study, the fish that received the contaminated diet also showed lower farming performances (FBW, FCR and SGR) compared to the control. These differences increased over time (Fig. 1, 2 and 3) and after 168 days of exposure to 367 µg kg^{-1} DON, fish that ingested DON presented a final weight of 487.40 g compared to 593.63 g in

the control group ($p = 0.053$, Fig. 1). However, these differences were never statistically significant.

A similar pattern of lower performance in the DON-fed animals was observed for FCR (Fig. 2) and SGR (Fig. 3): animals fed the control diet presented an FCR of 1.86 compared to 2.50 for DON-fed animals. PER was generally lower for animals that were fed DON and was significantly lower on day 92 ($p = 0.044$) and day 168 ($p = 0.050$; Table 4). Feed intake was generally higher for animals that were fed DON and was significantly higher on day 62 ($p = 0.041$; Table 5).

Histology

In the short-term exposure study, among the 2.7 DON groups, two out of ten animals showed mild to moderately hyalinized hepatocytes. In one trout, multiple areas of necrosis with scattered hemorrhages were present (Fig. 4). Vacuolation of hepatocytes was also more pronounced in 2.7 DON animals (5 out of 10 fish) compared to the control animals (no registered cases of vacuolation of hepatocytes). In the 1.1 DON groups, hyalinized hepatocytes were visible (6 out of 10 fish), but to a lesser extent compared with the 2.7 DON groups (8 out of 10 fish). No significant differences were obvious between any of the experimental groups based on counts of the mucous cell numbers in the intestinal mucosa, pigmented macrophage centers in the liver and kidney, and number of necrotic single cells in the liver. No histological alterations were found in the intestine or kidneys (head and trunk kidney).

Challenge test

Cumulative mortality after inoculation with *Y. ruckeri* is shown in Figure 5. The challenge trial lasted 17 days, and the 2.7 DON treatment showed a significantly higher survival rate ($p < 0.020$) compared to the control treatment. Controls exhibited 73.3% survival while the 1.1

DON and 2.7 DON treatments had a survival rate of 86.7% and 93.3%, respectively. No statistically significant differences were found between the 1.1 DON and 2.7 DON treatments or between the 1.1 DON treatment and the controls. The cause of death was confirmed as *Y. ruckeri* on the basis of the clinical signs. Furthermore, bacteria were re-isolated from the kidneys of infected fish. In each case, pure cultures were obtained, and the colonies displayed morphology consistent with *Y. ruckeri*. This was further confirmed by isolating the genomic DNA from selected colonies and performing PCR using the primers described by del Cerro *et al.* (2002). Fish that had recovered from the infection at the time of the challenge termination did not display any gross clinical signs. Similarly, non-infected fish did not display any signs of infection.

Liver Enzymes

Short-term DON exposure

The effects of the dietary treatments on LDH, ALT and AST activities in the serum are summarized in Table 6. Samples from the fish that received the dietary DON appeared to have a higher LDH activity, although these results were not statistically significant ($p = 0.078$). The 2.7 DON treatment showed a significant increase in ALT and AST activities (76.10 ± 9.88 IU L^{-1}; $p < 0.001$ and 876.50 ± 87.60 IU L^{-1}; $p < 0.001$, respectively) compared with the control (ALT = 14.20 ± 7.66 IU L^{-1} and AST = 389.70 ± 2.36 IU L^{-1}; Table 6).

Long-term DON exposure

Blood enzyme parameters measured at different sampling points are shown in Table 7. No significant differences were found during the experimental period for the different enzymes sampled.

Clinical signs

Few clinical signs were observed in the fish exposed to the mycotoxin, and when abnormalities were observed, only a small number of fish were affected. Among the abnormalities were abnormal body conformations, observed in 15 out of 60 fish that were fed 2,745 ± 330 µg kg^{-1} DON, characterized by a reduction in fish length in relation to width (Figure 6). In addition, in five out of 60 fish that were fed 2,745 ± 330 µg kg^{-1} DON, a protruding anal papilla was observed (Figure 7). Intussusceptions were observed internally in two fish.

Discussion

The decreasing supply and rising cost of fishmeal have led the aquaculture industry to investigate alternative sources of protein to substitute fishmeal in aquafeeds. Plant-based meals seem to be one of the most promising solutions for replacing fishmeal, and numerous plant raw materials have been successfully tested (Gatlin *et al.*, 2007). However, recent studies have noted the occurrence of mycotoxins in plant-based aquafeeds (Barbosa *et al.,* 2013, Pietsch *et al.*, 2013; Nácher-Mestre *et al.*, 2015; Gonçalves *et al.*, 2016; Gonçalves *et al.*, 2017, Greco *et al*., 2015). In the present study, the experimental diets were contaminated with several mycotoxins and fungal metabolites in addition to the added DON. The presence of other mycotoxins and fungal metabolites highlights the risk of mycotoxin contamination in aquaculture finished feeds. The present experimental diet represents a typical commercial trout diet that contains plant-based compounds (59.70% DM). The co-occurrence of mycotoxins and fungal metabolites in this diet, even at low concentrations, may lead to synergistic/additive/antagonistic effects between these compounds, which cannot be ruled out as a contributing factor for the obtained results. However, further studies are needed to address possible interactions between mycotoxins, especially at low contamination levels.

The objective of the present trial was to evaluate the possible effects of DON contamination in aquaculture feeds under two different scenarios. In the first scenario, the effect of short-term feeding of high levels of DON (50 days; 1,166 µg kg^{-1} DON and 2,745 µg kg^{-1} DON) was examined in an attempt to mimic the potential inclusion of highly contaminated raw material(s) in the finished feed. This situation would normally only affect a few batches of feed; therefore, the exposure would occur over a short period. In this scenario, the potential influence of mycotoxins on *Y. ruckeri* susceptibility was also evaluated. The second experiment studied the effects of long-term exposure to low levels of DON (168 days; 367 µg kg^{-1} DON). This experiment was designed to replicate a situation that is more commonly found because 367 µg kg^{-1} DON is comparable to the average DON contamination level previously found in aquafeeds during recent years (Gonçalves *et al*., 2016, 2017, and 2018).

One of the main constraints when researching mycotoxins in aquaculture species is the lack of mycotoxin-induced clinical symptoms. While it is true that several published reports describe some clinical signs for the most common mycotoxins (see the review conducted by Anater *et al.*, 2016), most of these clinical signs are very general and can be attributed to any other pathology or challenge faced by the animals, e.g., anti-nutrition factors or lectins in the diet (Hart *et al.*, 2010). Furthermore, the clinical signs typically present high variability.

In the present manuscript, the occurrence of clinical signs was evaluated in both the short- and long-term exposure experiments, and special attention was paid to visual clinical signs. In the short-term/high DON exposure experiment, 15 out of 60 fish that were fed 2,745 ± 330 µg kg^{-1} DON showed an abnormal body conformation, characterized by a fish length reduced in relation to its width, and five out of 60 fish from same treatment presented a protruding anal papilla. No clinical signs were observed after long-term exposure/low DON exposure. Although clinical manifestation was observed in a small number of individuals (only at the higher dosage of the short-term/high DON exposure experiment), it cannot be concluded that the signs observed are directly attributed to DON. The rectal prolapse observed in some fish is

also described as a DON clinical manifestation in swine when fed 3,000 µg kg^{-1} DON (Madson *et al.* 2014). However, a recent study (Gonçalves *et al.* 2018) stated that no macroscopic lesions were found (i.e., internal or external hemorrhages, dermal and oral lesions, abnormal pigmentation or damage to fins) on rainbow trout that were fed high levels of DON (11,412 ± 1141 µg kg^{-1}). Taking into account the previous study (Gonçalves *et al*. 2018) and three other studies with the same range of DON contamination (0.3 to 5.9 ppm), Hooft *et al*. (2011) and Ryerse *et al*. (2015) also reported no major pathological changes in the distal intestine of trout, while Matejova *et al*. (2014) found gastrointestinal hemorrhages. It is possible that the impact of DON might vary greatly depending on unknown factors, even for the same species.

Recently, Gonçalves *et al*. (2018) reported a novel DON metabolite (DON-3-sulfate) found in rainbow trout feces. The authors suggested that this biotransformation achieved by sulfation is probably realized by the trout gut microbiota as was previously described for other fish species (*Ameiurus nebulosus*; Guan *et al*., 2009). This biotransformation, if achieved by the gut microbiota, can also help to explain the high individual variability obtained, as the capacity to metabolize DON will be directly influenced by the individual fish microbiome. This explains the absence of clinical signs in some of the fish that were fed DON because DON-3-sulfate is less toxic than DON. The high inter-individual variation within the groups that were fed mycotoxins highlights the importance of the individual health and nutritional status prior to DON ingestion, as supported by other authors (Hendricks, 1994). Due to the reasons previous stated, the clinical manifestation found in the present study, even if only present in a small number of individuals, should be further confirmed as a DON exclusive clinical sign, associating it with an individual fish microbiome.

Reduction in feed intake is a well-documented response of rainbow trout to diets contaminated with naturally occurring or artificially added DON (Hooft *et al.*, 2011; Gonçalves *et al*. 2018; Ryerse *et al.*, 2015). In the present short-term study, fish that were fed

2,745 µg kg^{-1} of DON showed a significant reduction ($p < 0.001$) in feed intake. However, no effect was observed in fish that were fed 1,166 µg kg^{-1} of DON. A significant decrease in growth was also detected in the 2.7 DON treatment; TGC decreased by 17% ($p = 0.001$), and SGR decreased by 13% ($p < 0.001$). However, no significant differences ($p > 0.05$) were found for PER or FCR. In the long-term study, ingestion of DON was asymptomatic, as the animals presented no clinical signs, and growth rate was slightly affected only after 92 days of ingesting DON. At the end of the trial (168 days), the animals that were fed DON weighed less than the control animals. While not significantly different, the tendency for reduced weight gain in animals that were fed DON is consistent with the short-term experiment. Recently, Gonçalves *et al*. (2018) suggested that suppression of appetite due to DON contamination in feeds might be a defense mechanism to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON. The authors showed that PACAP (pituitary adenylate cyclase-activating polypeptide) seems to be fundamental for explaining the reduction of feed intake in DON-fed treatments, inducing anorexia, reinforcing the influence of DON on the hypothalamic melanocortin system. It is also important to mention that a contamination dose of 367 µg kg^{-1} of DON is a frequent and plausible level of contamination that is often found in aquafeeds incorporating plant meals (Gonçalves *et al*., 2016; Gonçalves *et al*., 2017). Moreover, this value is close to the limit of detection of most commercial ELISA (enzyme-linked immunosorbent assay) strip tests for DON, which means that samples need to be analyzed by more robust methods (e.g., HPLC), which increases costs and the time to receive sample results. The observed asymptomatic decrease in growth performance may lead to important economic consequences for the aquaculture industry.

In both experiments, it was difficult to correctly diagnose DON intake using the other parameters evaluated (liver enzymes and histology). In the short-term/high DON exposure study, histological and enzymatic changes showed different results, and individual variability was very high. Enzymatic activity was used to evaluate the possibility of tissue destruction.

ALT and AST have previously been used as markers of liver dysfunction (Gül *et al.*, 2004; Saravanan *et al.,* 2012), and ALT is an intracellular enzyme that has been used as a marker of tissue destruction in the liver. However, no clear pattern could be observed in the studies. Only in the short-term/high-level DON exposure study were elevated ALT serum levels found in the 2.7 DON treatment compared with the control group. In addition, AST values were significantly higher in the 2.7 DON treatment compared with the control. Elevated ALT and AST serum levels might be an indication of liver or other parenchymal organ damage. Liver histopathology revealed mild to moderate damage in a limited number of DON-exposed fish. However, no histological alterations were detected in the intestine or kidneys (head and trunk kidney). DON is known to cause impairment of barrier integrity, affecting the lamina propria and tight junctions, which may increase GIT permeability and consequently allow the entry of luminal antigens and bacteria normally restricted to the GIT lumen (Grenier *et al*., 2013, Dänicke *et al*., 2010). The fact that histological alterations were not found in the intestines, despite the altered values of ALT and AST, might lead us to hypothesize that short exposure periods might not be sufficient to lead to histological alterations and/or that histology might not be a good method to evaluate negative DON effects in the intestines. Moreover, as mentioned by Gonçalves *et al*. (2018), the individual microbiome seems to play an important role in DON biotransformation, which may also influence the obtained histological results. It would also be interesting to more closely examine the tight junction proteins as a more sensitive indicator for possible DON impact at the intestinal barrier, specifically at the tight junction level.

The results obtained for the *Y. ruckeri* challenge are consistent with the results from previous studies that investigated the effect of dietary DON on the mortality of rainbow trout challenged with other bacterial pathogens (Hooft *et al.* (2011) and Ryerse *et al.* (2015)). The apparent absence of an immunosuppressive effects on trout challenged with DON contrasts with published data for livestock species such as swine (Lessard *et al.*, 2015; Pierron *et al.*,

2016). An eventual direct suppression of *Y. ruckeri* by DON seems unlikely as it is very well described that trichothecenes interact with the eukaryotic 60S ribosomal subunit and prevent polypeptide chain initiation or elongation (Carter and Cannon, 1977; Ueno, 1984; Pestka, 2007). The present study did not include a pair-fed group (i.e., a group consuming the same amount of feed as that consumed by the DON groups), and thus it was not possible to distinguish the effects of feed restriction (caused by DON) from other effects of DON that might have decreased susceptibility to *Y. ruckeri.*

The intake of DON has been reported to lead to the upregulation of cytokine levels, especially pro-inflammatory cytokines (IL-6, IL-8 and IL-1β), in several studies (piglets, Bracarense *et al.* 2012); human intestinal Caco-2 cells (Maresca *et al.* 2008, Van De Walle *et al.* 2008); and mice (Azcona-Olivera *et al.* 1995)). Intestinal upregulation of pro-inflammatory cytokines may explain the higher resistance of DON-treated fish to infection with *Y. ruckeri*. However, as explained by Grenier and Applegate (2013), DON, as a protein synthesis inhibitor, might naturally originate superinduction phenomena, consequently increasing cytokine synthesis and secretion. Nonetheless, the possible role of DON in the upregulation of pro-inflammatory cytokines and the consequent effect on immune stimulation should be further investigated.

Conclusions

The present findings reinforce those from previous studies, concluding that the ingestion of DON by trout over short-term periods at high dosages (50 days; 1,166 µg kg^{-1} and 2,745 µg kg^{-1}) impacts growth performance, especially feed intake, with minor or variable biochemical changes in trout blood and histopathological changes. In this case, some fish did exhibit clinical symptoms (i.e., anal papilla), which could be attributed to the DON treatment; however, further confirmation is needed. This is the first report of the effects of the long-term exposure of rainbow trout to low concentrations of DON (168 days; 367 µg kg^{-1} DON). Ingestion of DON in the long-term study was asymptomatic; however, the fish started to

reduce their growth performance 92 days after ingesting DON. Such low contamination levels, which might be unnoticed by farmers, may have economic consequences for aquaculture.

DON-treated fish showed higher resistance to infection with *Y. ruckeri*, which may be related to stimulation of the pro-inflammatory response. While higher resistance to pathogen infection may be considered as a positive effect, the reduced feed intake and lower growth performance may have economic consequences for aquaculture. Moreover, further investigation is needed to understand the influence of DON on pro-inflammatory responses.

The high levels of individual variability observed in the blood biochemical parameters, histological changes and clinical signs in the fish that were fed DON might be explained by individual intestinal microbiota composition. The individual gut microbiome and its apparent capacity to metabolize DON should be further explored.

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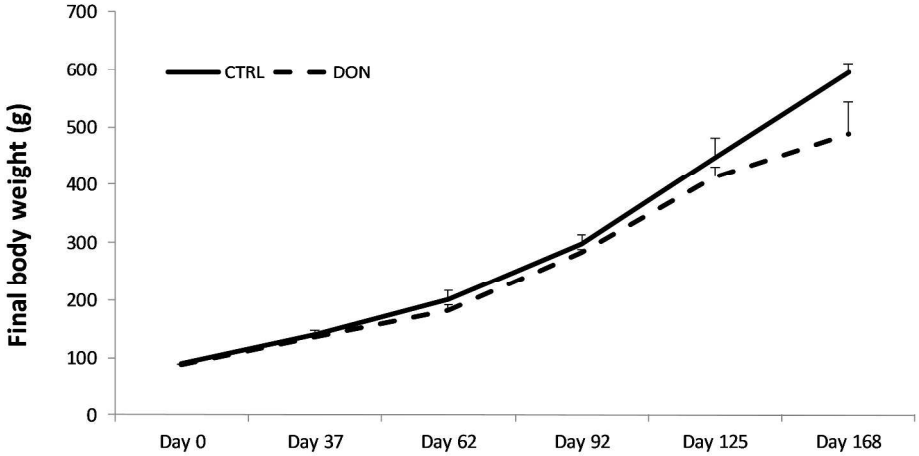

FIGURE 1: Growth curve representing the average weight of the fish during the long term experiment.

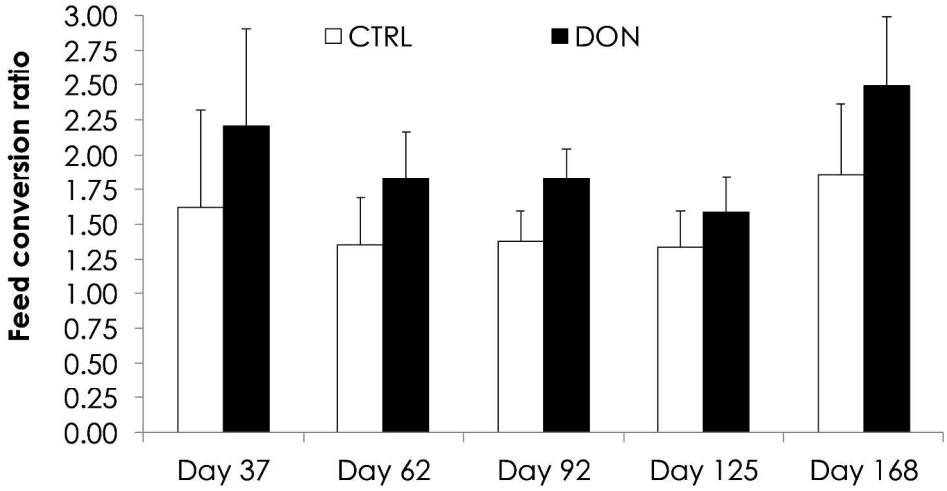

FIGURE 2: Feed conversion ratio at different sampling time points. Values are displayed as average ± standard deviation.

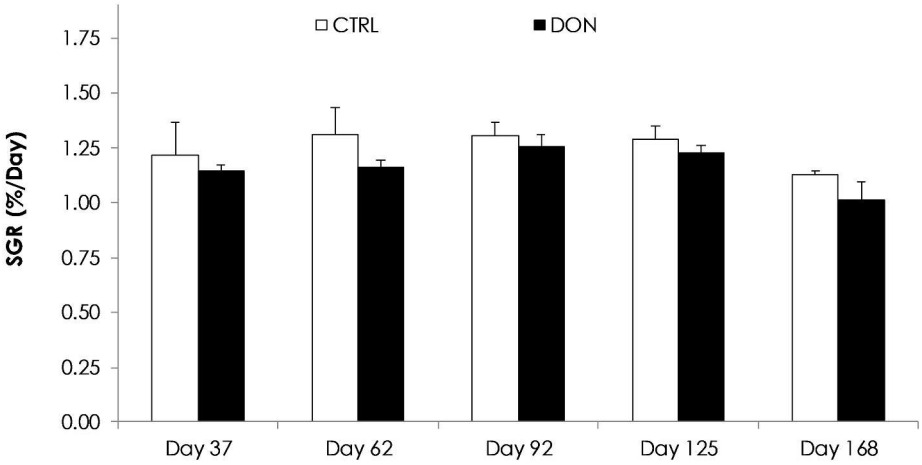

FIGURE 3: Specific growth rate at different sampling time points. Values are displayed as average ± standard deviation.

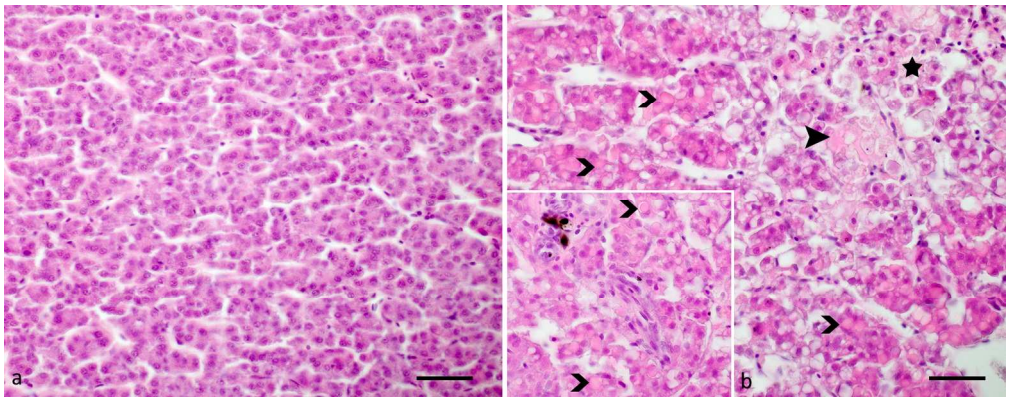

FIGURE 4. Onchorhynchus mykiss, histologic appearance of control (a) and 2.7 DON exposed fish (b); a. normal structure of hepatocytes; b. normal structure is disrupted, multiple hepatocytes are necrotic (star; observed in 1 out of 10 fishes sampled), scattered fibrin exudation (closed arrowhead; observed in 6 out of 10 fishes sampled), multiple hepatocytes show intracytoplasmatic eosinophilic, amorphous material (hyalinised hepatocytes) (open arrowheads; observed in 8 out of 10 fishes sampled), HE stain, bars = 50 µm; inlet: higher magnification showing hyalinised hepatocytes (open arrowheads).

180x70mm (300 x 300 DPI)

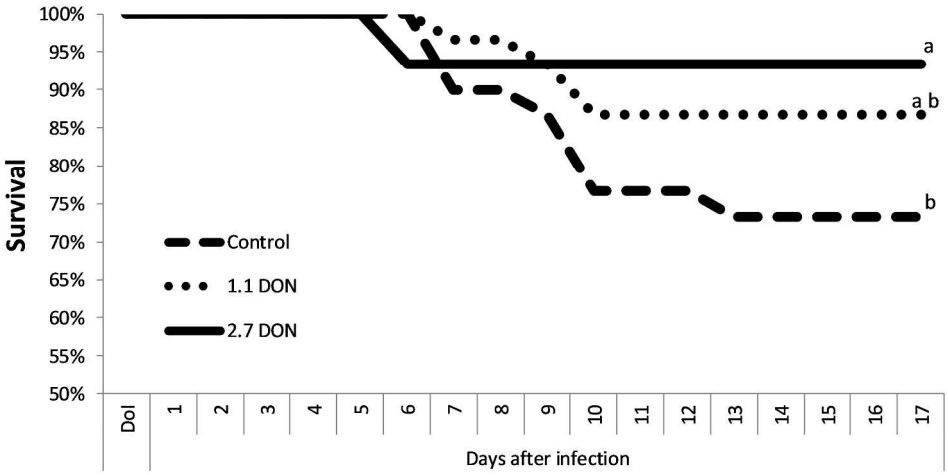

FIGURE 5. Survival curve following infection with Yersinia ruckeri during the high dose experiment.

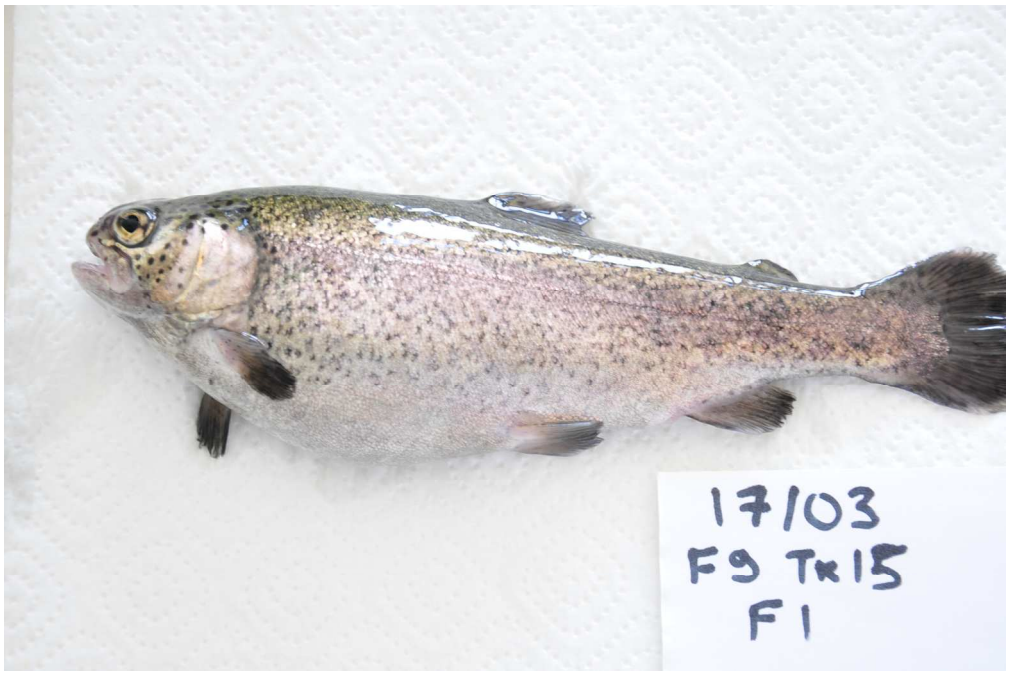

FIGURE 6. Abnormal body conformations, characterized by a fish length reduced in relation to its width. Observed in 15 fishes out of 60 fishes fed 2,745 ± 330 µg kg-1 DON.

181x120mm (300 x 300 DPI)

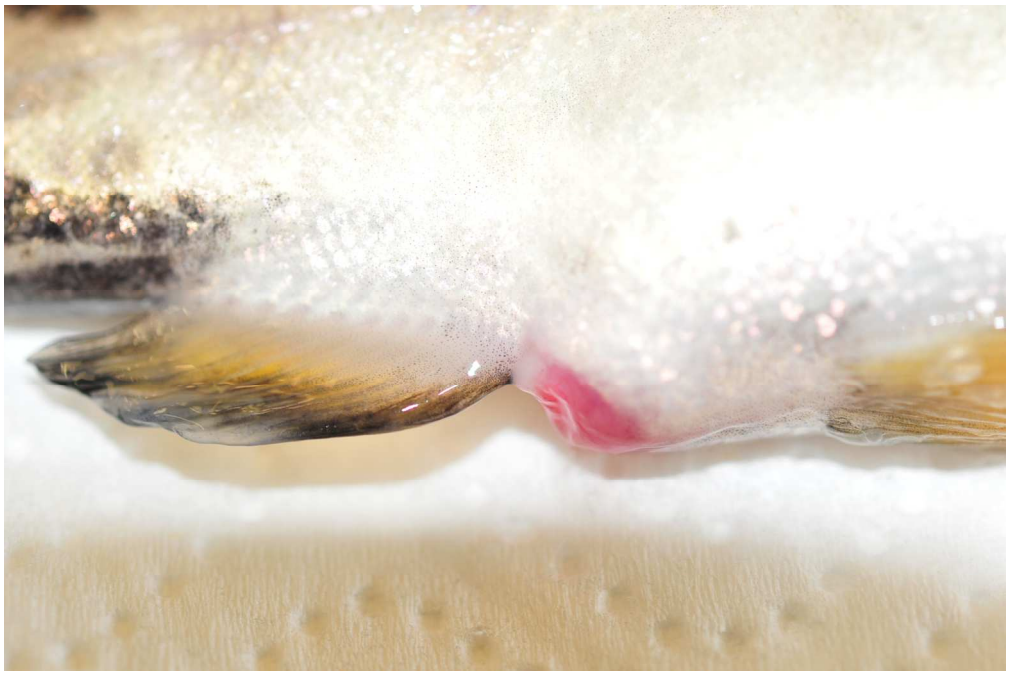

FIGURE 7. Fish presenting protruding anal papilla after being fed 2,745 ± 330 µg kg-1 DON. Observed in 5 fishes out of 60 fishes fed 2,745 ± 330 µg kg-1 DON.

181x120mm (300 x 300 DPI)

TABLE 1: Experimental control diet ingredients and proximate composition.

Ingredients	**CTRL**
	%
Fishmeal 60[a]	14.00
Fishmeal Super Prime[b]	12.45
Soy protein concentrate[c]	15.00
Wheat gluten[d]	12.30
Corn gluten meal[e]	8.00
Soybean meal[f]	6.00
Wheat meal[g]	6.40
Corn meal[h]	10.00
Fish oil[i]	10.00
Soy lecithin[j]	2.00
Antioxidant[k]	0.30
Monocalcium phosphate[l]	1.50
L-lysine[m]	0.50
DL-methionine[n]	0.50
Vitamin E[o]	0.05
Vitamin and mineral premix[p]	1.00
Proximate composition (%DM)	
Dry matter (DM), %	91.7 ± 0.0
Crude protein, % DM	52.2 ± 0.1
Crude fat, % DM	17.9 ± 0.0
Ash, % DM	9.3 ± 0.0
Gross energy, kJ/g DM	22.2 ± 0.0

[a] COFACO 60: 62.3% crude protein (CP), 8.4% crude fat (CF), COFACO, Portugal; [b] Super Prime: 67.4% CP, 8.2% CF, EXALMAR, Peru; [c] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; [d] VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France; [e] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; [f] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, CARGILL, Spain; [g] Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal; [h] Corn meal: 8.1% CP; 3.7% CF, Casa Lanchinha, Portugal; [i] SAVINOR, Portugal; [j] Lecico P700IPM, LECICO GmbH, Germany; [k] Paramega PX, Kemin Europe NV, Belgium; [l] MCP: 22% P, 18% Ca, Fosfitalia, Italy; [m] Lysine HCl 99%, Ajinomoto Eurolysine SAS, France; [n] DL-Methionine 99%, EVONIK DEGUSSA GmbH, Germany; [o] ROVIMIX E50, DSM Nutritional Products, Switzerland; [p] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

TABLE 2: Multi-mycotoxin analysis of experimental diets

Analyte	Concentration (µg kg^{-1})	Analyte	Concentration (µg kg^{-1})
Major mycotoxins		**Other *Fusarium* metabolites**	
Aflatoxin B1	<LOD	15-Hydroxyculmorin	48.33
Zearalenone	11.44	Culmorin	69.87
Deoxynivalenol	<LOD	Equisetin	10.39
Fumonisin B1	<LOD	Fusaric acid	65.56
Fumonisin B2	25.05	***Penicillium* metabolites**	
Fumonisin B4	16.11	Brevianamid F	194.30
Ochratoxin A	<LOD	Mycophenolic acid	88.91
Sum of Ergot alkaloids	0.72	Rugulusovin	244.20
		Other *Aspergillus* metabolites	
Zearalenone metabolites		Tryptophol	28.90
Zearalenone-sulfate	32.62	**Other metabolites**	
		Cyclo(L-Pro-L-Val)	1,631.00
		Cyclo(L-Pro-L-Tyr)	2,004.00

Deoxynivalenol	**target concentration**	**Analyzed concentration**
CTRL	0.0	0.0
1.1 DON	1,500	1,166 ± 140
2.7 DON	3,000	2,745 ± 330
0.3 DON	400	367 ± 66.80

Limits of detection (LOD) for AFB_1 = 0.3 µg kg^{-1}. For deoxynivalenol and ochratoxin A, detection limit are: 10, 50 and 0.2 µg kg−1 and for fumonisin B1 the detection limit are 25 µg kg^{-1}. Were analyzed 5 samples per diet.

TABLE 3: Growth performance parameters determined in the short term/high DON dosage study.

	Final Weight (g)	SGR (% day $^{-1}$)	PER	FI (g fish^{-1})	FCR	CF	TGC
CTRL	101.36 ± 19.81 [a]	2.52 ± 0.07 [a]	2.17 ± 0.05	81.21 ± 4.71 [a]	0.98 ± 0.07	1.42 ± 0.12 [ab]	0.113 ± 0.005 [a]
1.1 DON	95.37 ± 19.20 [a]	2.46 ± 0.06 [a]	2.01 ± 0.13	81.65 ± 3.78 [a]	1.03 ± 0.07	1.46 ± 0.13 [b]	0.109 ± 0.004 [a]
2.7 DON	79.91 ± 16.54 [b]	2.20 ± 0.09 [b]	2.01 ± 0.07	64.03 ± 2.87 [b]	1.05 ± 0.04	1.39 ± 0.12 [a]	0.094 ± 0.005 [b]
				1-way ANOVA			
p-value	<0.001	<0.001	0.096	<0.001	0.423	0.033	0.001

Data are presented as mean ± standard deviation. Values in the same column with different letters are significantly different ($P < 0.05$). NS = not significant. SGR = Specific growth rate; PER = Protein efficiency rate; FI = Feed intake; FCR = Feed conversion ratio; CF = Condition factor and TGC=thermal-unit growth coefficient.

	Day 37	Day 62	Day 92	Day 125	Day 168

CTRL	1.15 ± 0.17	1.38 ± 0.18	1.34 ± 0.11	1.38 ± 0.13	0.99 ± 0.03
0.3 DON	0.89 ± 0.22	1.03 ± 0.16	1.15 ± 0.14	1.18 ± 0.18	0.76 ± 0.14
			1-way ANOVA		
p-value	0.150	0.044	0.110	0.183	0.50

Values are displayed as mean ± standard deviation

TABLE 4: Protein efficiency rate at different sampling time points for the long term /low DON dosage experiment.

TABLE 5: Feed intake at different sampling time points for the long term /low DON dosage experiment.

	Day 37	**Day 62**	**Day 92**	**Day 125**	**Day 168**
CTRL	1.92 ± 0.06	1.67 ± 0.08	1.61 ± 0.07	1.43 ± 0.09	1.41 ± 0.03
0.3 DON	2.02 ± 0.08	1.85 ± 0.09	1.74 ± 0.10	1.59 ± 0.15	1.66 ± 0.68
			1-way ANOVA		
p-value	0.133	0.041	0.109	0.189	0.070

Values are displayed as mean ± standard deviation

TABLE 6: Effects of dietary treatments on LDH, ALT and AST activities in the serum for short term/high DON exposure experiment.

	LDH (IU L^{-1})	ALT (IU L^{-1})	AST (IU L^{-1})
CTRL	1000.60 ± 187.01[a]	14.20 ± 7.66[a]	389.70 ± 2.36[a]
1.1 DON	2001.18 ± 825.06[a]	22.00 ± 0.97[a]	543.80 ± 45.68[a]
2.7 DON	1700.60 ± 163.27[a]	76.10 ± 9.88[b]	876.50 ± 87.60[b]
1-way ANOVA p-value	0.078	<0.001	<0.001

Data are presented as mean ± SD. Values in the same column with different letters are significantly different ($P < 0.05$). LDH = Lactate Dehydrogenase; ALT = Alanine transaminase and AST = Aspartate Aminotransferase. (IU L^{-1}) = International Units per liter.

TABLE 7: Effects of dietary treatments on LDH, ALT, AST, ALP, Total protein and hematocrit in the serum at different sampling time points for long term/low DON exposure experiment.

	Sampling	Hematocrit (%)	ALT (IU L^{-1})	AST (IU L^{-1})	LDH (IU L^{-1})	ALP (IU L^{-1})	T-Prot. (g L^{-1})
CTRL	Initial	51.2±0.08	17.2±11.8	432.9±157.2	1846.5±1178.2	*	*
	62days	39.9±3.32	11.1±3.5	309.1±239.6	1862.7±1199.4	143.4±71.8	3.0±0.67
Mycotoxins		37.6±4.29	24.4±25.4	385.2±91.55	2497.0±1573.1	171.6±69.5	3.0±0.5
Control	125days	*	*	324.7±144.4	1968.7±1222.8	154.4±47.72	3.4±0.79
Mycotoxins		*	*	216.5±97.3	914.8±314.9	146.3±69.11	3.1±0.63

*Values could not be determined due to technical problems with samples. Values are displayed as averages ± standard deviation. N= 5 per treatment. LDH = Lactate dehydrogenase; ALT = Alanine transaminase and AST = Aspartate aminotransferase; ALP = Alkaline phosphatase; T-Prot.= Total protein. IU L^{-1} = International Units per liter.

Figure legend

FIGURE 1: Growth curve representing the average weight of the fish during the long term experiment.

FIGURE 2: Feed conversion ratio at different sampling time points. Values are displayed as average ± standard deviation.

FIGURE 3: Specific growth rate at different sampling time points. Values are displayed as average ± standard deviation.

FIGURE 4. *Onchorhynchus mykiss*, histologic appearance of control (a) and 2.7 DON exposed fish (b); a. normal structure of hepatocytes; b. normal structure is disrupted, multiple hepatocytes are necrotic (star; observed in 1 out of 10 fishes sampled), scattered fibrin exudation (closed arrowhead; observed in 6 out of 10 fishes sampled), multiple hepatocytes show intracytoplasmatic eosinophilic, amorphous material (hyalinised hepatocytes) (open arrowheads; observed in 8 out of 10 fishes sampled), HE stain, bars = 50 µm; inlet: higher magnification showing hyalinised hepatocytes (open arrowheads).

FIGURE 5. Survival curve following infection with *Yersinia ruckeri* during the high dose experiment.

FIGURE 6. Abnormal body conformations, characterized by a fish length reduced in relation to its width. Observed in 15 fishes out of 60 fishes fed 2,745 ± 330 µg kg^{-1} DON.

FIGURE 7. Fish presenting protruding anal papilla after being fed 2,745 ± 330 µg kg^{-1} DON. Observed in 5 fishes out of 60 fishes fed 2,745 ± 330 µg kg^{-1} DON.