



Effects of deoxynivalenol exposure time and contamination levels on rainbow trout

Journal:	<i>Journal of the World Aquaculture Society</i>
Manuscript ID	JWAS-18-032.R3
Manuscript Type:	Applied Studies
Date Submitted by the Author:	24-May-2018
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Keywords:	Mycotoxins, <i>Oncorhynchus mykiss</i> , pathogen susceptibility, hepatocytes hyalinization

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3 **1 Effects of deoxynivalenol exposure time and contamination levels on**
4
5 **2 rainbow trout**

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32 **14 Running title: Effects of deoxynivalenol on rainbow trout**

33
34
35
36 **16 Abstract**

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38 **17 The trend towards using plant-based ingredients in aquafeeds is set to intensify;**
39
40 **18 however, mycotoxin contamination might be a challenge. Two diets, with deoxynivalenol**
41
42 **19 (DON) levels of 1,166 µg kg⁻¹ (1.1 DON) and 2,745 µg kg⁻¹ (2.7 DON), were prepared for**
43
44 **20 short-term DON-exposure (50 days). A third diet with a low DON level of 367 µg kg⁻¹**
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46 **21 (0.3 DON) was prepared for long-term DON-exposure (168 days). Ingestion of DON by**
47
48 **22 trout during both short-term/high-dosage exposure (50 days; 1,166 µg kg⁻¹ and 2,700 µg**
49
50 **23 kg⁻¹ DON) and long-term/low-dosage exposure (168 days; 367 µg kg⁻¹ DON) impacted**
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52 **24 growth performance and, to a lesser extent, liver enzyme parameters (2.7 DON).**
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55 **25 Histopathology showed mild to moderate changes in the liver but not in the other**

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3 26 **sampled tissues (intestine and kidney). Despite these effects, short-term exposure of**
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5 27 **rainbow trout to high doses of DON did not result in increased susceptibility to *Yersinia***
6
7 28 ***ruckeri*. In both the short- and long-term studies, the effects of DON showed a high**
8
9 29 **inter-individual variability. The present study confirms that sub-clinical levels of**
10
11 30 **mycotoxins affect rainbow trout. The effects of such low mycotoxin levels could be**
12
13 31 **masked by other production challenges while still negatively affecting productivity.**
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20 34 *Keywords:* Mycotoxins, *Oncorhynchus mykiss*, pathogen susceptibility, hepatocytes
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22 35 hyalinization
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39 **Introduction**

39 In aquaculture, the trend to replace expensive animal-derived proteins, such as fishmeal, with
40 more economical and sustainable plant protein sources has increased the probability of
41 mycotoxin contamination in aquaculture feeds. According to Tacon *et al.* (2011), plant-based
42 ingredients already represent the major dietary protein source used in feeds for lower trophic
43 level fish species, such as tilapia, carp and catfish. These ingredients also account for the
44 second major source of dietary protein and lipids after fishmeal and fish oil in the feed of
45 shrimp and high trophic level fish species. Various plant sources have been used for
46 salmonids but at lower inclusion levels than feed destined for lower trophic species. In most
47 aquaculture species, plant protein choice and selection are based on a combination of local
48 market availability, cost and the nutritional profile (including anti-nutrient content and level)
49 of the plant meal in question (Gatlin *et al.*, 2007; Davis and Sookying, 2009; Krogdahl *et al.*,
50 2010).

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3 51 The mycotoxin contamination of finished feeds and raw materials used in aquaculture as well
4
5 52 as the negative effects of mycotoxins on aquatic species, particularly rainbow trout
6
7 53 (*Oncorhynchus mykiss*), has been highlighted in recent publications (Hooft *et al.*, 2011;
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9 54 Ryerse *et al.*, 2015; Tola *et al.*, 2015; Gonçalves *et al.*, 2018; Hooft and Bureau, 2017).
10
11 55 However, mycotoxin contamination is not generally assessed in commercial aquafeeds or
12
13 56 plant meals used to manufacture these feeds. Consequently, we do not have accurate estimates
14
15 57 of the mycotoxin contamination levels in these commodities.

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18 58 Few studies are currently available concerning mycotoxin occurrence in aquaculture plant
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20 59 meals and finished feeds. Gonçalves *et al.* (2016) reported that deoxynivalenol (DON) was
21
22 60 present in 68% of analyzed samples (shrimp and fish, sampled in Asia and Europe in 2014) at
23
24 61 average contamination levels of 162 $\mu\text{g kg}^{-1}$ and maximum levels of 413 $\mu\text{g kg}^{-1}$. More
25
26 62 recently, Gonçalves *et al.* (2018) observed that contamination patterns for shrimp and fish
27
28 63 feeds were slightly different, which likely reflects the type of commodity used for the
29
30 64 different species. The authors observed that shrimp feeds were generally contaminated with
31
32 65 low levels of DON, with the exception of some diets (contamination ranging from 329 $\mu\text{g kg}^{-1}$
33
34 66 to 2,287 $\mu\text{g kg}^{-1}$ of DON). In the case of fish feeds, samples were contaminated mainly by
35
36 67 DON, up to a maximum level of 396 $\mu\text{g kg}^{-1}$, and were co-contaminated with other
37
38 68 mycotoxins.

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40
41 69 Trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis, interfering
42
43 70 with the initiation, elongation, and termination stages of this process (Kumar *et al.*, 2013).
44
45 71 Knowledge of the effects of DON on aquatic species has increased recently (Hooft *et al.*,
46
47 72 2011; Matejova *et al.*, 2015; Ryerse *et al.*, 2015; Tola *et al.*, 2015; Hooft and Bureau, 2017;
48
49 73 Gonçalves *et al.*, 2018), and studies on rainbow trout suggest that DON has a detrimental
50
51 74 effect on feed intake, weight gain and feed efficiency (Hooft *et al.*, 2011; Ryerse *et al.*, 2015).
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53 75 Curiously, no effect has been detected on the immune status of animals fed with DON
54
55 76 (Matejova *et al.*, 2015; Matejova *et al.*, 2017; Ryerse *et al.*, 2015).

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3 77 In general, the effects of mycotoxins vary greatly depending on a variety of factors,
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5 78 including nutritional and health status prior to exposure, dose and duration of exposure, age,
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7 79 species and infection route. In addition, the lack of reliable clinical signs or parameters
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9 80 (including biomarkers) to correctly diagnose the ingestion of DON by aquatic species makes
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11 81 mycotoxin risk management in aquaculture very challenging.

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13 82 The aim of the present study was to evaluate the effect of DON on rainbow trout under two
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15 83 different scenarios: first, the effect of short-term feeding of high levels of DON (50 days;
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17 84 1,166 $\mu\text{g kg}^{-1}$ DON and 2,745 $\mu\text{g kg}^{-1}$ DON), and second, the effects of long-term feeding of
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19 85 low levels of DON (168 days; 367 $\mu\text{g kg}^{-1}$ DON). Moreover, we aimed to investigate the
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21 86 manifestation of clinical signs due to the ingestion of DON by inspecting several organs and
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23 87 tissues normally affected by the consumption of mycotoxins.
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29 **Materials and methods**

30 *Experimental diets*

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33 91 The experimental diets were formulated to be isoenergetic (22.20 kJ g^{-1} dry matter (DM),
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35 92 isoproteic (52.20% DM) and isolipidic (17.90% DM) (Table 1)). All diets were formulated
36
37 93 with the same ingredients. Marine-derived ingredients (fishmeal and fish oil) represented
38
39 94 22.45% DM of the diet, whereas plant raw materials represented 59.70% DM of the diet. All
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41 95 ingredients were finely ground (hammer mill, 0.8-mm sieve), mixed, and then extruded (twin
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43 96 screw extruder, 2.0-mm pellet size, SPAROS, Portugal).

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45
46 97 The ingredients used to formulate the diets were subjected to Liquid chromatography-tandem
47
48 98 mass spectrometry, HPLC-MS/MS-based multi-mycotoxin analysis (University of Natural
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50 99 Resources and Life Sciences, Center for Analytical Chemistry Department IFA, Austria), as
51
52 100 described by Streit *et al.* (2013). The method covered major type A and B trichothecenes,
53
54 101 zearalenone, fumonisins, aflatoxins and ochratoxins. For the purpose of data analysis, non-
55
56 102 detect levels were based on the limits of detection (LOD) of the method used for analysis. The

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2
3 103 detected concentrations of major mycotoxins and of a selection of other fungal metabolites are
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5 104 listed in Table 2.

6
7 105 Diets with three different levels of DON were prepared by adding DON (Romer Labs
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9 106 Diagnostic GmbH, Austria) to the feed during diet ingredient mixing. Two diets, with DON
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11 107 levels of 1,166 $\mu\text{g kg}^{-1}$ (1.1 DON) and 2,745 $\mu\text{g kg}^{-1}$ (2.7 DON), were prepared for short-term
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13 108 DON exposure (50 days). A third diet with a low DON level of 367 $\mu\text{g kg}^{-1}$ (0.3 DON) was
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15 109 prepared for long-term DON exposure (168 days). All diets were dried at 45 C for 12 hours
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17 110 after the addition of DON and were stored at 4 C until use.

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20 111 Contamination levels were chosen taking into account previous literature on the effect of
21
22 112 DON on rainbow trout (Hooft, Elmor *et al.*, 2011; Matejova *et al.*, 2014; Matejova, Vicenova
23
24 113 *et al.*, 2015; Ryerse, Hooft *et al.*, 2015) as well as the reported DON levels in worldwide
25
26 114 finished feed samples (Gonçalves *et al.*, 2016, 2017, 2018; Greco *et al.*, 2015; Barbosa *et al.*,
27
28 115 2013). The long-term exposure to DON attempts to mimic the most recently reported levels of
29
30 116 DON in finished feeds (Gonçalves *et al.*, 2018, average of 82.87 $\mu\text{g kg}^{-1}$ and maximum of 396
31
32 117 $\mu\text{g kg}^{-1}$). However, the authors are aware that reports of mycotoxin occurrence in European
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34 118 aquaculture finished feeds are still very limited, and levels reported may vary annually (e.g.,
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36 119 average DON contamination of 160.86 $\mu\text{g kg}^{-1}$ in 2014, of 165.61 $\mu\text{g kg}^{-1}$ in 2015, and of
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38 120 87.87 $\mu\text{g kg}^{-1}$ in 2016; Gonçalves *et al.*, 2016, 2017 and 2018). Generally, Asian aquafeed
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40 121 samples present higher DON levels compared with European aquafeed samples.
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43 44 45 46 123 *Fish and experimental conditions*

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48 124 This study was approved by the institutional ethics committee and the national authority
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50 125 according to §26 of Law for Animal Experiments, Tierversuchsgesetz 2016—TVG 2012
51
52 126 under No. BMFWF- 68.205/0135-WF/V/3b/2014. Rainbow trout (*Oncorhynchus mykiss*)
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54 127 originating from a farm with no prior history of *Yersiniosis* was used in both experiments. On
55
56 128 arrival, the kidneys of ten fish were sampled, and their infection-free status was confirmed by

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3 129 culture-based analysis and polymerase chain reaction (PCR)-based analysis using *Yersinia*
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5 130 *ruckeri* specific primers (del Cerro *et al.*, 2002).
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7 131 *Short-term exposure to DON*
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9 132 For the experiment with short-term exposure to DON, 180 fish (14.10 ± 0.05 g) were
10
11 133 randomly allocated to three feeding groups in quadruplicate and given either standard feed
12
13 134 (control, CTRL), feed contaminated with $1,166 \mu\text{g kg}^{-1}$ DON (1.1 DON) or feed contaminated
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15 135 with $2,745 \mu\text{g kg}^{-1}$ DON (2.7 DON). Each aquarium of 85 L was supplied by a flow-through
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17 136 system with a temperature of 15.47 ± 0.14 C, oxygen concentration of 8.73 ± 0.12 mg L⁻¹, and
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19 137 pH of 7.53 ± 0.04 , with 0.0 ± 0.0 mg L⁻¹ total ammonia nitrogen, nitrites and nitrates. The fish
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21 138 were hand-fed the prepared diets (CTRL, 1.1 DON or 2.7 DON) three times per day near
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23 139 satiety for 50 days prior to performing the *Y. ruckeri* challenge.
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28 141 *Long-term exposure to DON*
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31 142 For the long-term exposure experiment, 120 fish weighing 89 ± 8 g were randomly allocated
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33 143 and distributed among eight tanks, each with a volume of 1 m³, supplied by a flow-through
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35 144 system with a water temperature of 18.6 ± 1.0 C, oxygen concentration of 8.56 ± 0.26 mg L⁻¹
36
37 145 and pH of 7.35 ± 0.35 . Each tank contained 15 fish that were fed restrictively (2.5% of the
38
39 146 average body mass) with either control feed (CTRL, 4 tanks) or the control feed supplemented
40
41 147 with $367 \mu\text{g kg}^{-1}$ DON (0.3 DON, 4 tanks) for 168 days. The same quantity of feed (2.5% of
42
43 148 the average body mass) was distributed in each tank by hand feeding and was adjusted after
44
45 149 intermediate weighing periods (at 37, 62, 92 and 125 days). Five fish per replicate tank were
46
47 150 subjected to moderate anesthesia (tricaine methanesulfonate (MS222) (Sigma-Aldrich Co.,
48
49 151 LLC, Bellfonte, USA) at a dose of 0.7 g L⁻¹, and a blood sample was collected by puncture of
50
51 152 the caudal vein with a heparinized syringe at the beginning of the trial and at 62 and 125 days.
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53 153 Part of the blood sample was used for the determination of hematocrit, which was determined
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55 154 for five fish per treatment. Blood was transferred into hematocrit capillary tubes
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3 155 (Hirschmann), the tubes were then centrifuged at 13,000 RPM for 5 minutes (Hettich
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5 156 Haematokrit 200), and the percentage of red blood cells to sera was measured. The remaining
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7 157 part of the blood sample was centrifuged at 1,590 x g for ten minutes, after which the plasma
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9 158 (i.e., the supernatant fraction) was transferred to Eppendorf tubes, snap-frozen in liquid
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11 159 nitrogen and stored at -80 C until subsequent analysis of total protein. Total protein was
12
13 160 determined by the Bradford method (Bradford 1976) using bovine serum albumin as the
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15 161 standard. All measurements were performed in a Synergy HT multi-mode microplate reader
16
17 162 (BIOTEK, Vermont, USA).
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22 164 *Growth performance*

23
24 165 All fish, in both the short- and long-term exposure experiments, were weighed to determine
25
26 166 the initial individual body weight at the start of the experiments. In the short-term exposure
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28 167 study, the fish were weighed individually at the end of the 50-day period, and their total
29
30 168 length was measured and recorded. Feed intake was recorded daily. In the long-term exposure
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32 169 study, the fish were weighed individually after 37, 62, 92, 125 and 168 days.
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34

35 170 The following calculations were made in both experiments.
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37 171 The thermal-unit growth coefficient (TGC) was expressed as the growth rate and was
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39 172 calculated for each aquarium as $[100 \times (\text{FBW } 1/3 - \text{IBW } 1/3) / \Sigma (\text{Temp } (^{\circ}\text{C}) \times \text{number of}$
40
41 173 $\text{days})]$, where FBW = final body weight (g fish⁻¹) and IBW = initial body weight (g fish⁻¹).
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43

44 174 The feed conversion ratio (FCR) was calculated as crude feed intake/weight gain, where FI =
45
46 175 total dry feed/number of fish.
47

48 176 The protein efficiency ratio (PER) was calculated as weight gain (g)/protein intake (g).
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50 177 The specific growth rate (SGR) was calculated as $[(\ln \text{ final weight} - \ln \text{ initial weight})/\text{time in}$
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52 178 $\text{days}] \times 100$.
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3 179 Fulton's condition factor, K , was also used to measure individual fish health: $K =$
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5 180 $100(BW/L^3)$, where BW is the whole body wet weight (g) and L is the length (cm). A factor
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7 181 of 100 was used to transform K to approximate a value of one.
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11 183 *Liver enzymes*

12
13 184 In the short-term/high DON exposure experiment, five fish from each aquarium were sampled
14
15 185 at the end of the experiment (50 days) for analysis of liver enzymes in blood. In the long-
16
17 186 term/low DON exposure study, five fish from each aquarium were sampled on day 62 and on
18
19 187 day 125. The fish were anesthetized by immersion in tricaine methanesulfonate (MS222)
20
21 188 (Sigma-Aldrich Co., LLC, Bellfonte, USA) at a dose of 0.7 g L^{-1} prior to blood collection.
22
23 189 Blood samples were analyzed to measure the activities of lactate dehydrogenase (LDH),
24
25 190 alanine transaminase (ALT) and aspartate aminotransferase (AST) using a Spotchem EZ SP-
26
27 191 4430 reader and Spotchem II GPT/ALT, Spotchem II LDH and Spotchem II GOT/AST kits
28
29 192 (all products from Arkay, Amstelveen, Netherlands).
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35 194 *Histological examination*

36
37 195 For the short-term/high DON exposure study, organs were sampled from 10 fish prior to the
38
39 196 *Y. ruckeri* challenge and at the time of termination. The intestine, spleen, liver and kidneys
40
41 197 (head and trunk kidney) of the fish were removed and fixed in 10% buffered formalin for 48
42
43 198 to 72 hours. The samples were embedded overnight in paraffin using a HistoMaster
44
45 199 (Formafix, Düsseldorf, Germany). Sections (3 - 4 μm thick) were cut from each paraffin
46
47 200 block and were left to dry overnight at 37 C before being stained with hematoxylin and eosin.
48
49 201 The slides were evaluated under a light microscope (Nikon Eclipse E400, Feasterville,
50
51 202 Pennsylvania). The following were examined: intestine (number of mucous cells in mucosa),
52
53 203 liver (hepatocyte vacuolation, hepatocyte hyalinization, single cell necrosis, number of
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55 204 pigmented macrophage centers, perivascular and peribiliary inflammation), and kidney
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3 205 (number of pigmented macrophage centers). To evaluate the number of cells, three high-
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5 206 power fields (HPF) were counted per slide.
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9 208 *Bacterial preparation*

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11 209 As a pre-trial to the challenge test, five groups of ten fish each were challenged by
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13 210 immersion with *Y. ruckeri* isolate 7959/11 to determine the appropriate infectious dose. *Y.*
14
15 211 *ruckeri* isolate A7959/11 is a clinical isolate that originated from an outbreak at an Austrian
16
17 212 trout farm in 2011. This isolate was kept at -80 C on beads until three days prior to the start of
18
19 213 the experiment. It was then inoculated on a blood agar plate and incubated at 22 C. After 48
20
21 214 hours, a single colony was inoculated into 7.5 ml of BHI broth and was incubated in a shaking
22
23 215 incubator at 20 C with rotation at 150 rpm. After 10 hours, the cultures were evaluated by eye,
24
25 216 and 2.5 ml was sampled from one culture and used to inoculate a 1.5-L BHI broth. This broth
26
27 217 was then incubated for approximately 12 hours at 20 C with shaking at 150 rpm.
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33 219 *Infection trial in the short-term exposure study*

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35 220 After 50 days, each feeding group of the short-term/high DON exposure study was further
36
37 221 divided into two groups: two of the aquaria were infected with *Y. ruckeri* while fish in the two
38
39 222 other aquaria were mock-infected with un-inoculated broth. In total, 90 fish were infected and
40
41 223 90 were mock-infected. The infection procedure was adapted from that described for
42
43 224 *Aeromonas salmonicida* (Menanteau-Ledouble *et al.*, 2017). Briefly, bacteria were grown
44
45 225 overnight in 1.5 L of BHI broth and their concentration was determined by measuring the
46
47 226 optical density at a wavelength of 600 nm (OD600) per ml. Water circulation in the aquarium
48
49 227 was interrupted, and the water volume was lowered to 50 L. The bacterial culture (2 ml) was
50
51 228 added to each of the aquaria, yielding a final concentration of 2×10^4 CFU mL⁻¹. The fish
52
53 229 remained in the solution for two hours, after which the water was progressively returned to its
54
55 230 normal level and the circulation was reopened. The fish were monitored at least twice daily.
56

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2
3 231 Mortalities were recorded, and dead and moribund fish were immediately removed from the
4
5 232 tanks. Moribund fish were euthanatized by prolonged immersion in a solution of 1 g L⁻¹ of
6
7 233 MS-222, and the kidney of the fish was sampled for microbial re-isolation of the pathogen on
8
9 234 an agar plate. The colonies growing on these plates were examined and confirmed to be *Y.*
10
11 235 *ruckeri* based on their morphologies. Furthermore, one in five isolates was selected; its
12
13 236 genomic DNA was isolated using a Qiagen DNeasy kit, and PCR was performed using *Y.*
14
15 237 *ruckeri* specific primers (del Cerro *et al.*, 2002). The surviving fish overcame the infection 17
16
17 238 days post-infection, at which point the challenge was terminated. All remaining fish were
18
19 239 euthanatized by prolonged immersion in a solution of tricaine methanesulfonate (MS222; 1 g
20
21 240 L⁻¹ of water), weighed, measured and examined for gross clinical signs of enteric red mouth
22
23 241 syndrome (oral congestion, hemorrhages or petechia, exophthalmia and ocular hemorrhages,
24
25 242 ascites in the abdominal cavity, enlarged spleens and hemorrhages or petechia in the internal
26
27 243 organs, bloody intestines or adipose tissues).
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29
30
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32 33 245 *Clinical signs*

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35 246 During both experiments, gross clinical signs were assessed by visual examination of the fish
36
37 247 at the time of termination. Lesions (hemorrhages and ulcerations) on the skin were recorded,
38
39 248 as were any obvious abnormalities such as a protruding anal papilla. The state of the gills was
40
41 249 recorded as well as the presence of anemia, hemorrhages or necrosis.

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43
44 250 The fish were examined internally for any abnormalities. In particular, record was made of
45
46 251 congestions, petechia or hemorrhages of the internal organs. The color of the liver and the size
47
48 252 of the spleen were assessed, as was the general health of the intestine (in particular, the
49
50 253 presence of congestion, hemorrhage or intussusception was determined).
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55 56 57 *Statistical analysis*

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3 257 All parameters such as the final weight, SGR, PER, FI, FCR, condition factor (CF), TGC,
4
5 258 LDH, ALT and AST were subjected to analysis of variance in SPSS 21 for Windows (IBM
6
7 259 Corp., Armonk, NY, USA). One-way ANOVA was performed, and differences between the
8
9 260 means were tested by Tukey's multiple range test. The Shapiro-Wilk test was used to analyze
10
11 261 the normality, and homogeneity of variances was tested using Levene's test. Data analyzed
12
13 262 did not violate the assumption of equal variances and showed a normal distribution. All
14
15 263 parameters expressed as percentages were subjected to arcsin square root transformation.
16
17 264 Additionally, one-way ANOVA was performed to analyze the histological differences in the
18
19 265 intestine (number of mucous cells in mucosa) and liver (single cell necrosis, number of
20
21 266 pigmented macrophage centers, perivascular and peribiliary inflammation) between the DON
22
23 267 dietary treatments and controls.
24
25
26 268 Following the challenge, survival curves were constructed for each treatment, and Kaplan-
27
28 269 Meier and odds ratio analyses were performed using SPSS v.20 (IBM) and MedCalc
29
30 270 (Microsoft).
31
32
33 271 The level of significance was set at $p < 0.05$, and the results are presented as the mean \pm SD
34
35 272 (standard deviation of the mean).
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38

274 **Results**

275 *Experimental diets*

276 The four experimental diets were formulated to be isoenergetic (22.20 kJ g⁻¹ DM), isoproteic
277 (52.20% DM) and isolipidic (17.90% DM) and to meet all the nutrient requirements for the
278 species examined in the study. There was no significant difference ($p > 0.05$) between
279 treatments regarding the nutritional composition of the experimental diets. Analysis of the
280 feed to confirm mycotoxin levels showed DON contamination was successfully achieved,
281 although observed levels were slightly lower than intended (Table 2). Other
282 metabolites/toxins were found in the basal diet (common to all experimental groups) due to

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

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Growth performance

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Short-term DON exposure

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The results showed that rainbow trout was sensitive to the DON levels tested (Table 3).

293

The presence of $2,700 \mu\text{g kg}^{-1}$ DON in the diet led to a significant decrease ($p < 0.001$) in FI.

294

The same treatment (2.7 DON) also resulted in a significant decrease in the final weight

295

($79.91 \pm 16.54 \text{ g}$; $p < 0.001$), SGR ($2.20 \pm 0.09\% \text{ day}^{-1}$; $p < 0.001$), TGC (0.094 ± 0.005 ; $p <$

296

0.001) and CF (1.39 ± 0.12 ; $p < 0.033$) compared to the controls (final weight = 101.36 ± 19.8

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g ; SGR = $2.52 \pm 0.07\% \text{ day}^{-1}$; TGC = 0.113 ± 0.005 and CF = 1.42 ± 0.12). Observations of

298

the feeding behavior of the DON-fed groups confirmed that the fish initially accepted the

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feed, and a reduction in FI was progressively established. We therefore assumed that the

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lower FI in the DON-fed groups compared to the control group was probably not due to the

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unfavorable organoleptic properties of DON-contaminated feed.

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303

Long-term DON exposure

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In the long-term exposure study, the fish that received the contaminated diet also showed

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lower farming performances (FBW, FCR and SGR) compared to the control. These

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differences increased over time (Fig. 1, 2 and 3) and after 168 days of exposure to $367 \mu\text{g kg}^{-1}$

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DON, fish that ingested DON presented a final weight of 487.40 g compared to 593.63 g in

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3 308 the control group ($p = 0.053$, Fig. 1). However, these differences were never statistically
4
5 309 significant.

6
7 310 A similar pattern of lower performance in the DON-fed animals was observed for FCR (Fig.
8
9 311 2) and SGR (Fig. 3): animals fed the control diet presented an FCR of 1.86 compared to 2.50
10
11 312 for DON-fed animals. PER was generally lower for animals that were fed DON and was
12
13 313 significantly lower on day 92 ($p = 0.044$) and day 168 ($p = 0.050$; Table 4). Feed intake was
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15 314 generally higher for animals that were fed DON and was significantly higher on day 62 ($p =$
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17 315 0.041 ; Table 5).
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Histology

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23
24 318 In the short-term exposure study, among the 2.7 DON groups, two out of ten animals showed
25
26 319 mild to moderately hyalinized hepatocytes. In one trout, multiple areas of necrosis with
27
28 320 scattered hemorrhages were present (Fig. 4). Vacuolation of hepatocytes was also more
29
30 321 pronounced in 2.7 DON animals (5 out of 10 fish) compared to the control animals (no
31
32 322 registered cases of vacuolation of hepatocytes). In the 1.1 DON groups, hyalinized
33
34 323 hepatocytes were visible (6 out of 10 fish), but to a lesser extent compared with the 2.7 DON
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36 324 groups (8 out of 10 fish). No significant differences were obvious between any of the
37
38 325 experimental groups based on counts of the mucous cell numbers in the intestinal mucosa,
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40 326 pigmented macrophage centers in the liver and kidney, and number of necrotic single cells in
41
42 327 the liver. No histological alterations were found in the intestine or kidneys (head and trunk
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44 328 kidney).
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Challenge test

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52 331 Cumulative mortality after inoculation with *Y. ruckeri* is shown in Figure 5. The challenge
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54 332 trial lasted 17 days, and the 2.7 DON treatment showed a significantly higher survival rate (p
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56 333 < 0.020) compared to the control treatment. Controls exhibited 73.3% survival while the 1.1
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3 334 DON and 2.7 DON treatments had a survival rate of 86.7% and 93.3%, respectively. No
4
5 335 statistically significant differences were found between the 1.1 DON and 2.7 DON treatments
6
7 336 or between the 1.1 DON treatment and the controls. The cause of death was confirmed as *Y.*
8
9 337 *ruckeri* on the basis of the clinical signs. Furthermore, bacteria were re-isolated from the
10
11 338 kidneys of infected fish. In each case, pure cultures were obtained, and the colonies displayed
12
13 339 morphology consistent with *Y. ruckeri*. This was further confirmed by isolating the genomic
14
15 340 DNA from selected colonies and performing PCR using the primers described by del Cerro *et*
16
17 341 *al.* (2002). Fish that had recovered from the infection at the time of the challenge termination
18
19 342 did not display any gross clinical signs. Similarly, non-infected fish did not display any signs
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21 343 of infection.
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26 345 *Liver Enzymes*

28 346 *Short-term DON exposure*

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31 347 The effects of the dietary treatments on LDH, ALT and AST activities in the serum are
32
33 348 summarized in Table 6. Samples from the fish that received the dietary DON appeared to
34
35 349 have a higher LDH activity, although these results were not statistically significant ($p =$
36
37 350 0.078). The 2.7 DON treatment showed a significant increase in ALT and AST activities
38
39 351 ($76.10 \pm 9.88 \text{ IU L}^{-1}$; $p < 0.001$ and $876.50 \pm 87.60 \text{ IU L}^{-1}$; $p < 0.001$, respectively) compared
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41 352 with the control (ALT = $14.20 \pm 7.66 \text{ IU L}^{-1}$ and AST = $389.70 \pm 2.36 \text{ IU L}^{-1}$; Table 6).
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46 354 *Long-term DON exposure*

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48 355 Blood enzyme parameters measured at different sampling points are shown in Table 7. No
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50 356 significant differences were found during the experimental period for the different enzymes
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52 357 sampled.
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Clinical signs

360 Few clinical signs were observed in the fish exposed to the mycotoxin, and when
361 abnormalities were observed, only a small number of fish were affected. Among the
362 abnormalities were abnormal body conformations, observed in 15 out of 60 fish that were fed
363 $2,745 \pm 330 \mu\text{g kg}^{-1}$ DON, characterized by a reduction in fish length in relation to width
364 (Figure 6). In addition, in five out of 60 fish that were fed $2,745 \pm 330 \mu\text{g kg}^{-1}$ DON, a
365 protruding anal papilla was observed (Figure 7). Intussusceptions were observed internally in
366 two fish.

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Discussion

369 The decreasing supply and rising cost of fishmeal have led the aquaculture industry to
370 investigate alternative sources of protein to substitute fishmeal in aquafeeds. Plant-based
371 meals seem to be one of the most promising solutions for replacing fishmeal, and numerous
372 plant raw materials have been successfully tested (Gatlin *et al.*, 2007). However, recent
373 studies have noted the occurrence of mycotoxins in plant-based aquafeeds (Barbosa *et al.*,
374 2013, Pietsch *et al.*, 2013; Nacher-Mestre *et al.*, 2015; Gonçalves *et al.*, 2016; Gonçalves *et*
375 *al.*, 2017, Greco *et al.*, 2015). In the present study, the experimental diets were contaminated
376 with several mycotoxins and fungal metabolites in addition to the added DON. The presence
377 of other mycotoxins and fungal metabolites highlights the risk of mycotoxin contamination in
378 aquaculture finished feeds. The present experimental diet represents a typical commercial
379 trout diet that contains plant-based compounds (59.70% DM). The co-occurrence of
380 mycotoxins and fungal metabolites in this diet, even at low concentrations, may lead to
381 synergistic/additive/antagonistic effects between these compounds, which cannot be ruled out
382 as a contributing factor for the obtained results. However, further studies are needed to
383 address possible interactions between mycotoxins, especially at low contamination levels.

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3 384 The objective of the present trial was to evaluate the possible effects of DON contamination
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5 385 in aquaculture feeds under two different scenarios. In the first scenario, the effect of short-
6
7 386 term feeding of high levels of DON (50 days; 1,166 $\mu\text{g kg}^{-1}$ DON and 2,745 $\mu\text{g kg}^{-1}$ DON)
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9 387 was examined in an attempt to mimic the potential inclusion of highly contaminated raw
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11 388 material(s) in the finished feed. This situation would normally only affect a few batches of
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13 389 feed; therefore, the exposure would occur over a short period. In this scenario, the potential
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15 390 influence of mycotoxins on *Y. ruckeri* susceptibility was also evaluated. The second
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17 391 experiment studied the effects of long-term exposure to low levels of DON (168 days; 367 μg
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19 392 kg^{-1} DON). This experiment was designed to replicate a situation that is more commonly
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21 393 found because 367 $\mu\text{g kg}^{-1}$ DON is comparable to the average DON contamination level
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23 394 previously found in aquafeeds during recent years (Gonçalves *et al.*, 2016, 2017, and 2018).
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25 395 One of the main constraints when researching mycotoxins in aquaculture species is the lack of
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27 396 mycotoxin-induced clinical symptoms. While it is true that several published reports describe
28
29 397 some clinical signs for the most common mycotoxins (see the review conducted by Anater *et*
30
31 398 *al.*, 2016), most of these clinical signs are very general and can be attributed to any other
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33 399 pathology or challenge faced by the animals, e.g., anti-nutrition factors or lectins in the diet
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35 400 (Hart *et al.*, 2010). Furthermore, the clinical signs typically present high variability.
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37 401 In the present manuscript, the occurrence of clinical signs was evaluated in both the short- and
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39 402 long-term exposure experiments, and special attention was paid to visual clinical signs. In the
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41 403 short-term/high DON exposure experiment, 15 out of 60 fish that were fed 2,745 \pm 330 $\mu\text{g kg}^{-1}$
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43 404 DON showed an abnormal body conformation, characterized by a fish length reduced in
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45 405 relation to its width, and five out of 60 fish from same treatment presented a protruding anal
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47 406 papilla. No clinical signs were observed after long-term exposure/low DON exposure.
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49 407 Although clinical manifestation was observed in a small number of individuals (only at the
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51 408 higher dosage of the short-term/high DON exposure experiment), it cannot be concluded that
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53 409 the signs observed are directly attributed to DON. The rectal prolapse observed in some fish is
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3 410 also described as a DON clinical manifestation in swine when fed 3,000 $\mu\text{g kg}^{-1}$ DON
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5 411 (Madson *et al.* 2014). However, a recent study (Gonçalves *et al.* 2018) stated that no
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7 412 macroscopic lesions were found (i.e., internal or external hemorrhages, dermal and oral
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9 413 lesions, abnormal pigmentation or damage to fins) on rainbow trout that were fed high levels
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11 414 of DON ($11,412 \pm 1141 \mu\text{g kg}^{-1}$). Taking into account the previous study (Gonçalves *et al.*
12
13 415 2018) and three other studies with the same range of DON contamination (0.3 to 5.9 ppm),
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15 416 Hooft *et al.* (2011) and Ryerse *et al.* (2015) also reported no major pathological changes in
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17 417 the distal intestine of trout, while Matejova *et al.* (2014) found gastrointestinal hemorrhages.
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19 418 It is possible that the impact of DON might vary greatly depending on unknown factors, even
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21 419 for the same species.

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24 420 Recently, Gonçalves *et al.* (2018) reported a novel DON metabolite (DON-3-sulfate) found in
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26 421 rainbow trout feces. The authors suggested that this biotransformation achieved by sulfation is
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28 422 probably realized by the trout gut microbiota as was previously described for other fish
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30 423 species (*Ameiurus nebulosus*; Guan *et al.*, 2009). This biotransformation, if achieved by the
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32 424 gut microbiota, can also help to explain the high individual variability obtained, as the
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34 425 capacity to metabolize DON will be directly influenced by the individual fish microbiome.
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36 426 This explains the absence of clinical signs in some of the fish that were fed DON because
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38 427 DON-3-sulfate is less toxic than DON. The high inter-individual variation within the groups
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40 428 that were fed mycotoxins highlights the importance of the individual health and nutritional
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42 429 status prior to DON ingestion, as supported by other authors (Hendricks, 1994). Due to the
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44 430 reasons previous stated, the clinical manifestation found in the present study, even if only
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46 431 present in a small number of individuals, should be further confirmed as a DON exclusive
47
48 432 clinical sign, associating it with an individual fish microbiome.

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51 433 Reduction in feed intake is a well-documented response of rainbow trout to diets
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53 434 contaminated with naturally occurring or artificially added DON (Hooft *et al.*, 2011;
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55 435 Gonçalves *et al.* 2018; Ryerse *et al.*, 2015). In the present short-term study, fish that were fed

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3 436 2,745 $\mu\text{g kg}^{-1}$ of DON showed a significant reduction ($p < 0.001$) in feed intake. However, no
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5 437 effect was observed in fish that were fed 1,166 $\mu\text{g kg}^{-1}$ of DON. A significant decrease in
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7 438 growth was also detected in the 2.7 DON treatment; TGC decreased by 17% ($p = 0.001$), and
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9 439 SGR decreased by 13% ($p < 0.001$). However, no significant differences ($p > 0.05$) were
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11 440 found for PER or FCR. In the long-term study, ingestion of DON was asymptomatic, as the
12
13 441 animals presented no clinical signs, and growth rate was slightly affected only after 92 days of
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15 442 ingesting DON. At the end of the trial (168 days), the animals that were fed DON weighed
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17 443 less than the control animals. While not significantly different, the tendency for reduced
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19 444 weight gain in animals that were fed DON is consistent with the short-term experiment.
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21 445 Recently, Gonçalves *et al.* (2018) suggested that suppression of appetite due to DON
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23 446 contamination in feeds might be a defense mechanism to decrease the exposure of the animal
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25 447 to DON, therefore reducing the potential negative impacts of DON. The authors showed that
26
27 448 PACAP (pituitary adenylate cyclase-activating polypeptide) seems to be fundamental for
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29 449 explaining the reduction of feed intake in DON-fed treatments, inducing anorexia, reinforcing
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31 450 the influence of DON on the hypothalamic melanocortin system. It is also important to
32
33 451 mention that a contamination dose of 367 $\mu\text{g kg}^{-1}$ of DON is a frequent and plausible level of
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35 452 contamination that is often found in aquafeeds incorporating plant meals (Gonçalves *et al.*,
36
37 453 2016; Gonçalves *et al.*, 2017). Moreover, this value is close to the limit of detection of most
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39 454 commercial ELISA (enzyme-linked immunosorbent assay) strip tests for DON, which means
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41 455 that samples need to be analyzed by more robust methods (e.g., HPLC), which increases costs
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43 456 and the time to receive sample results. The observed asymptomatic decrease in growth
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45 457 performance may lead to important economic consequences for the aquaculture industry.
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47 458 In both experiments, it was difficult to correctly diagnose DON intake using the other
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49 459 parameters evaluated (liver enzymes and histology). In the short-term/high DON exposure
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51 460 study, histological and enzymatic changes showed different results, and individual variability
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53 461 was very high. Enzymatic activity was used to evaluate the possibility of tissue destruction.
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3 462 ALT and AST have previously been used as markers of liver dysfunction (Gül *et al.*, 2004;
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5 463 Saravanan *et al.*, 2012), and ALT is an intracellular enzyme that has been used as a marker of
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7 464 tissue destruction in the liver. However, no clear pattern could be observed in the studies.
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9 465 Only in the short-term/high-level DON exposure study were elevated ALT serum levels found
10
11 466 in the 2.7 DON treatment compared with the control group. In addition, AST values were
12
13 467 significantly higher in the 2.7 DON treatment compared with the control. Elevated ALT and
14
15 468 AST serum levels might be an indication of liver or other parenchymal organ damage. Liver
16
17 469 histopathology revealed mild to moderate damage in a limited number of DON-exposed fish.
18
19 470 However, no histological alterations were detected in the intestine or kidneys (head and trunk
20
21 471 kidney). DON is known to cause impairment of barrier integrity, affecting the lamina propria
22
23 472 and tight junctions, which may increase GIT permeability and consequently allow the entry of
24
25 473 luminal antigens and bacteria normally restricted to the GIT lumen (Grenier *et al.*, 2013,
26
27 474 Dänicke *et al.*, 2010). The fact that histological alterations were not found in the intestines,
28
29 475 despite the altered values of ALT and AST, might lead us to hypothesize that short exposure
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31 476 periods might not be sufficient to lead to histological alterations and/or that histology might
32
33 477 not be a good method to evaluate negative DON effects in the intestines. Moreover, as
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35 478 mentioned by Gonçalves *et al.* (2018), the individual microbiome seems to play an important
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37 479 role in DON biotransformation, which may also influence the obtained histological results. It
38
39 480 would also be interesting to more closely examine the tight junction proteins as a more
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41 481 sensitive indicator for possible DON impact at the intestinal barrier, specifically at the tight
42
43 482 junction level.

44
45 483 The results obtained for the *Y. ruckeri* challenge are consistent with the results from previous
46
47 484 studies that investigated the effect of dietary DON on the mortality of rainbow trout
48
49 485 challenged with other bacterial pathogens (Hooft *et al.* (2011) and Ryerse *et al.* (2015)). The
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51 486 apparent absence of an immunosuppressive effects on trout challenged with DON contrasts
52
53 487 with published data for livestock species such as swine (Lessard *et al.*, 2015; Pierron *et al.*,

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3 488 2016). An eventual direct suppression of *Y. ruckeri* by DON seems unlikely as it is very well
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5 489 described that trichothecenes interact with the eukaryotic 60S ribosomal subunit and prevent
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7 490 polypeptide chain initiation or elongation (Carter and Cannon, 1977; Ueno, 1984; Pestka,
8
9 491 2007). The present study did not include a pair-fed group (i.e., a group consuming the same
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11 492 amount of feed as that consumed by the DON groups), and thus it was not possible to
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13 493 distinguish the effects of feed restriction (caused by DON) from other effects of DON that
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15 494 might have decreased susceptibility to *Y. ruckeri*.

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18 495 The intake of DON has been reported to lead to the upregulation of cytokine levels, especially
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20 496 pro-inflammatory cytokines (IL-6, IL-8 and IL-1 β), in several studies (piglets, Bracarense *et*
21
22 497 *al.* 2012); human intestinal Caco-2 cells (Maresca *et al.* 2008, Van De Walle *et al.* 2008); and
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24 498 mice (Azcona-Olivera *et al.* 1995)). Intestinal upregulation of pro-inflammatory cytokines
25
26 499 may explain the higher resistance of DON-treated fish to infection with *Y. ruckeri*. However,
27
28 500 as explained by Grenier and Applegate (2013), DON, as a protein synthesis inhibitor, might
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30 501 naturally originate superinduction phenomena, consequently increasing cytokine synthesis
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32 502 and secretion. Nonetheless, the possible role of DON in the upregulation of pro-inflammatory
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34 503 cytokines and the consequent effect on immune stimulation should be further investigated.
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37 504

505 **Conclusions**

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42 506 The present findings reinforce those from previous studies, concluding that the ingestion of
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44 507 DON by trout over short-term periods at high dosages (50 days; 1,166 $\mu\text{g kg}^{-1}$ and 2,745 μg
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46 508 kg^{-1}) impacts growth performance, especially feed intake, with minor or variable biochemical
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48 509 changes in trout blood and histopathological changes. In this case, some fish did exhibit
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50 510 clinical symptoms (i.e., anal papilla), which could be attributed to the DON treatment;
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52 511 however, further confirmation is needed. This is the first report of the effects of the long-term
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54 512 exposure of rainbow trout to low concentrations of DON (168 days; 367 $\mu\text{g kg}^{-1}$ DON).
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56 513 Ingestion of DON in the long-term study was asymptomatic; however, the fish started to

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3 514 reduce their growth performance 92 days after ingesting DON. Such low contamination
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5 515 levels, which might be unnoticed by farmers, may have economic consequences for
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7 516 aquaculture.

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9 517 DON-treated fish showed higher resistance to infection with *Y. ruckeri*, which may be related
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11 518 to stimulation of the pro-inflammatory response. While higher resistance to pathogen
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13 519 infection may be considered as a positive effect, the reduced feed intake and lower growth
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15 520 performance may have economic consequences for aquaculture. Moreover, further
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17 521 investigation is needed to understand the influence of DON on pro-inflammatory responses.

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19 522 The high levels of individual variability observed in the blood biochemical parameters,
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21 523 histological changes and clinical signs in the fish that were fed DON might be explained by
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23 524 individual intestinal microbiota composition. The individual gut microbiome and its apparent
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25 525 capacity to metabolize DON should be further explored.
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33 Acknowledgments

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35 529 We thank many of our colleagues at BIOMIN, especially Dr. Dian Schatzmayr, Dr.
36
37 530 Christiane Gruber-Dorninger and Caroline Noonan, and our colleagues at the University of
38
39 531 Veterinary Medicine for helpful discussions and critical review of the manuscript.
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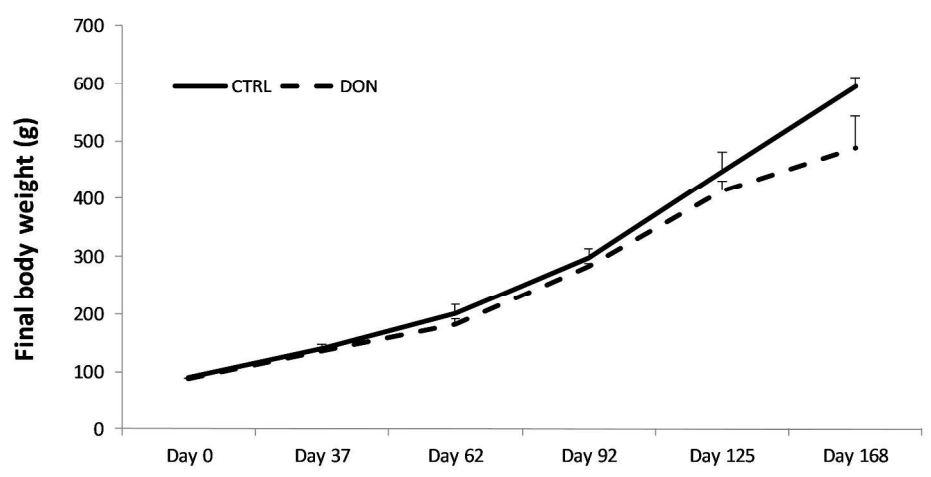


FIGURE 1: Growth curve representing the average weight of the fish during the long term experiment.

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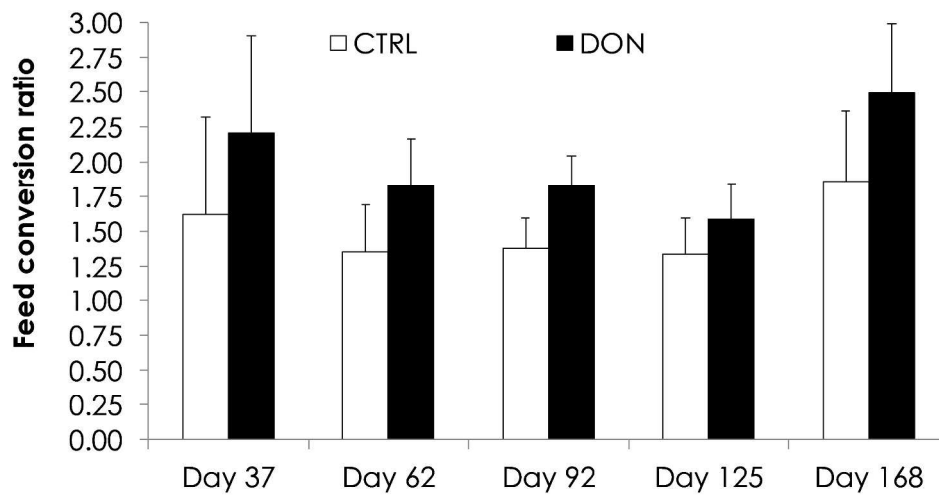


FIGURE 2: Feed conversion ratio at different sampling time points. Values are displayed as average \pm standard deviation.

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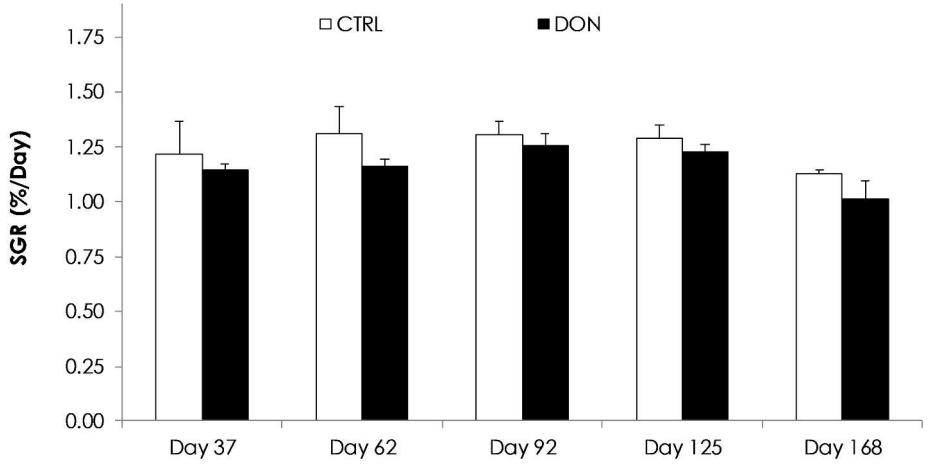


FIGURE 3: Specific growth rate at different sampling time points. Values are displayed as average \pm standard deviation.

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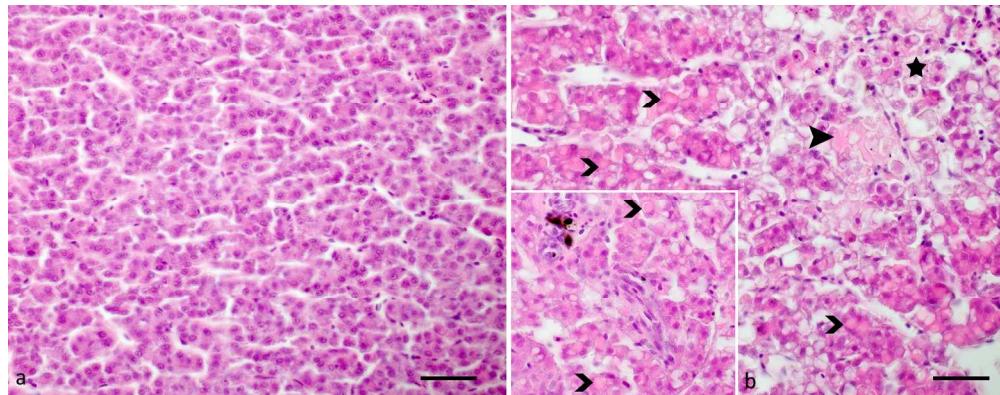


FIGURE 4. *Onchorhynchus mykiss*, histologic appearance of control (a) and 2.7 DON exposed fish (b); a. normal structure of hepatocytes; b. normal structure is disrupted, multiple hepatocytes are necrotic (star; observed in 1 out of 10 fishes sampled), scattered fibrin exudation (closed arrowhead; observed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

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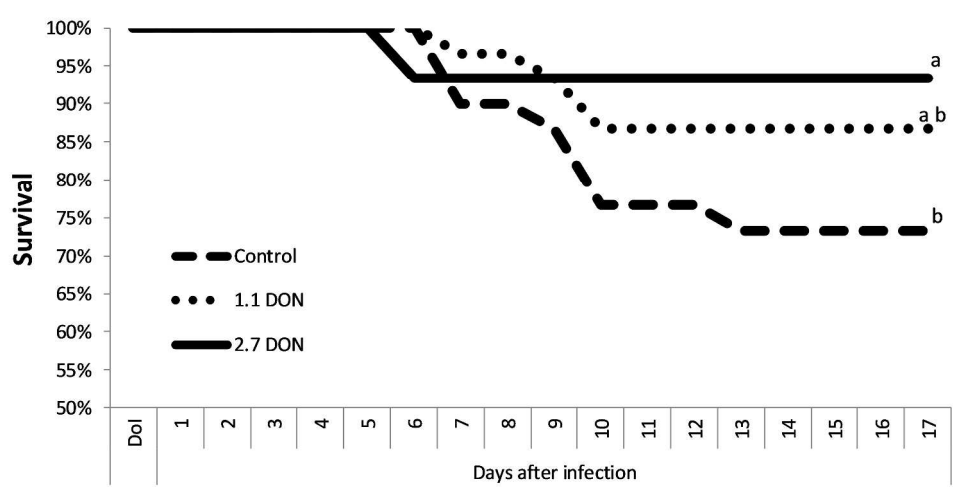


FIGURE 5. Survival curve following infection with *Yersinia ruckeri* during the high dose experiment.



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 $2,745 \pm 330 \mu\text{g kg}^{-1}$ DON.

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FIGURE 7. Fish presenting protruding anal papilla after being fed $2,745 \pm 330 \mu\text{g kg}^{-1}$ DON. Observed in 5 fishes out of 60 fishes fed $2,745 \pm 330 \mu\text{g kg}^{-1}$ DON.

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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 ($\mu\text{g kg}^{-1}$)	Analyte	Concentration ($\mu\text{g kg}^{-1}$)
Major mycotoxins		Other <i>Fusarium</i> 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	<i>Penicillium</i> 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
Zearalenone metabolites		Other <i>Aspergillus</i> metabolites	
Zearalenone-sulfate	32.62	Tryptophol	28.90
		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 \pm 140	
2.7 DON	3,000	2,745 \pm 330	
0.3 DON	400	367 \pm 66.80	

Limits of detection (LOD) for AFB₁ = 0.3 $\mu\text{g kg}^{-1}$. For deoxynivalenol and ochratoxin A, detection limit are: 10, 50 and 0.2 $\mu\text{g kg}^{-1}$ and for fumonisin B1 the detection limit are 25 $\mu\text{g kg}^{-1}$. 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.

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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.

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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)
	Initial	51.2±0.08	17.2±11.8	432.9±157.2	1846.5±1178.2	*	*
CTRL	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.

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