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Are the effects of Deoxynivalenol (DON) on performance, liver and gastrointestinal tract health of rainbow trout (*Oncorhynchus mykiss*) influenced by dietary composition?

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ABSTRACT

This study investigated if dietary composition influences the effects of Deoxynivalenol (DON) on the health and performance of rainbow trout (*Oncorhynchus mykiss*). Four experimental diets (2 × 2 factorial design) were formulated which differed in 1) the diet composition; fishmeal (FM) versus soybean meal-based (SBM) and 2) the DON content of wheat; clean versus naturally contaminated wheat. Triplicate groups of n = 30 fish were assigned to each diet: (1) CON-FM; DON= 0 μg/kg feed; (2) DON-FM; DON= 1200 μg/kg feed; (3) CON-SBM; DON= 46 μg/kg feed; (4) DON-SBM; DON= 1300 μg/kg feed. The 8 week experiment was divided into two feeding periods: after 6 weeks of restrictive feeding, fish were fed *ad libitum* for 2 weeks. Influences on performance were evaluated by determining growth, protein and energy gain metrics, and on health parameters through the determination of histopathological changes in the liver and gastrointestinal tract (GIT). Restrictive feeding showed negative effects of DON and dietary composition on performance but did not show an interaction between DON and diet composition. Similarly, subsequent *ad libitum* feeding showed effects of DON and/or diet composition on growth, feed efficiency and body biometrics, but no interaction effects. These data confirmed the challenging nature of the SBM-based diet and previously noted negative effects of DON on performance. Only the histopathological assessment of mucosal fold width, enterocyte width and goblet cell density indicated significant interaction effects between DON and diet composition in the midgut. Yet, the differences were generally small and interaction effects were restricted to the midgut and not to the hindgut which is usually challenged by SBM. The combined information on growth performance and health suggests that DON challenges rainbow trout regardless of being fed a FM- or SBM-based diet, allowing more flexible formulations in the aquafeeds.

1. Introduction

Globally, the growth of the aquaculture sector strongly depends on the expansion of aquafeed production (51 Mt in 2017 and 73 Mt expected in 2025) (Naylor et al., 2021; Tacon, 2020). Consequently, the ingredient composition of aquafeeds has diversified, with more plant- and animal-based ingredients/by-products of terrestrial origin being included in novel formulae (Naylor et al., 2021). For instance, in Norwegian salmon feeds, the inclusion level of marine ingredients has declined from 89% in 1990 to 41% in 2010 (Ytrestøyl et al., 2015) and even further to 25% in 2016 (Aas et al., 2019) and 22% in 2020 (Aas et al., 2022). Apart from nutritional imbalances, the increasing use of plant-based ingredients is linked to the introduction of anti-nutritional factors, contaminants and mycotoxins (Francis et al., 2001). Due to climate change, environmental conditions might become more favourable for fungus development on crops, leading to an increase in fungus-derived mycotoxin occurrence in plant-based ingredients which could be transferred to finished aquafeeds (Anater et al., 2016; Gonçalves et al., 2020; Koletsi et al., 2021).

Ever since mycotoxins have been described as emerging feed

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contaminants for European aquaculture, it has become evident that deoxynivalenol (DON) is the most prevalent mycotoxin in aquafeeds (Koletsi et al., 2021). Compared to other fish species, rainbow trout (*Oncorhynchus mykiss*) is very sensitive to DON (Hooft et al., 2011; Koletsi et al., 2021). Despite the sensitivity of fish to DON, comparatively few studies have investigated the impacts of DON on fish (Hooft and Bureau, 2021), in stark contrast to the depth of research on this topic in terrestrial animals. However, a meta-analysis of the available data across several fish species show that feed intake and growth decline exponentially with dietary DON levels (Koletsi et al., 2021). In more detail, DON has been shown, among others, to reduce protein gain in rainbow trout which suggest an inhibition of protein synthesis (Koletsi et al., 2022). DON has been reported to induce liver pathology in several fish species, as indicated by marked alterations in hepatic histological parameters (Hooft et al., 2011; Koletsi et al., 2022; Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2014). Gut health is also impacted through the reduction in expression of genes that regulate tight junction proteins in grass carp, indicating a disruption of the intestinal epithelial barrier (Huang et al., 2018). In rainbow trout, the available literature suggests that DON impacts fish health predominantly by inducing changes in the liver with less impact on the intestinal barrier integrity, as evidenced by minimal changes in intestinal histological parameters (Koletsi et al., 2022). Experimentally, studies on the effects of DON have generally targeted the concentration of the mycotoxin instead of focusing on the composition of the diet. Although studies with DON in carnivorous fish have hitherto been performed against a background of optimal quality marine-based diets.

Traditionally, soybean meal (SBM) is the most frequently used plant feedstuff to replace fishmeal in aquafeeds (Oliva-Teles et al., 2022). However, SBM can be challenging for fish, as it can contain antinutritional factors, such as soy saponins (Oliva-Teles et al., 2015), suggested to be the primary inducers of intestinal enteritis in Atlantic salmon (Krogdahl et al., 2015), and its inclusion level is thus often restricted in salmonid diets. Symptoms of SBM-induced enteritis in the distal intestine were first described for Atlantic salmon (Krogdahl et al., 2003; van den Ingh et al., 1991; van den Ingh et al., 1996), but later also for rainbow trout, which seem to endure slightly higher SBM levels before developing enteritis (Heikkinen et al., 2006; Merrifield et al., 2009; Mosberian-Tanha et al., 2016; Romarheim et al., 2008). Symptoms of SBM-induced enteritis in rainbow trout also include increased permeability of the distal intestinal epithelium, which may lead to reduced nutrient uptake and affect performance (Merrifield et al., 2009; Mosberian-Tanha et al., 2016; Nordrum et al., 2000). Enteritis-associated changes such as induced by SBM could possibly influence mycotoxin-induced effects on fish performance and health. Therefore, SBM is often used in nutritional challenge models in salmonid fish species.

The main aim of this study was to assess if dietary composition influences the effect of DON on rainbow trout. Based on the findings that SBM can lead to the disruption of the integrity of the intestinal epithelium in salmonids (Knudsen et al., 2008; Mosberian-Tanha et al., 2016), we hypothesised that the combination of DON and a challenging SBM-based diet could aggravate effects of DON in rainbow trout, in particular with respect to the functioning of the gastrointestinal tract (GIT). To this end, we designed both an 'optimal quality' marine-based diet and a 'sub-optimal quality' SBM-based diet with and without dietary DON. The effects of dietary DON were investigated through a detailed assessment of both performance and health metrics of rainbow trout fed these experimental diets.

2. Materials and methods

This experiment was carried out at the experimental facilities of the Alltech Coppens Aqua Centre (Leende, The Netherlands). The project (number AVD2330020198084) had been approved by the Central Committee on Animal Experiments (CCD) of The Netherlands and all experimental procedures were carried out in accordance with the Dutch law on the use of animals for scientific purposes.

2.1. In vivo experimental procedure

Experimental methods, system and husbandry procedures, duration of exposure, feeding regimes and practices, and sampling protocols were similar to those described for an earlier study (Koletsi et al., 2022). Briefly, 10 g rainbow trout were obtained from a commercial trout farm (Mohnen Aquaculture GmbH, Germany) and acclimatized for one week feeding a standard commercial trout diet. Groups of 30 fish were each randomly stocked in one of 12 120-L tanks (triplicates per dietary treatment) of a recirculating aquaculture system (RAS). Throughout the experiment, water temperature was kept constant at 14 ± 0.5 °C, and a photoperiod of 17 h of light and 7 h of darkness was used. The following water physicochemical parameters were monitored and kept within optimal ranges: pH: 7.0–8.5, NH_4^+: < 1 mg/L, NO_2^-: < 0.5 mg/L, and oxygen (O_2) above 8 mg/L.

2.2. Experimental diets

Four experimental diets were formulated according to a 2 × 2 factorial design (Table 1). The first factor aimed to create a contrast in the "quality" of the diets, which was done by replacing 25% of an optimal quality fishmeal LT (FM) by 25% non-GMO soybean meal (SBM; CP >45). Therefore, the diets were not nutritionally identical (iso-energetic and isonitrogenous). The lower crude protein (38,8) and lipid (14,7) content of SBM compared to FM (44,6 and 17, respectively) was not compensated in order to generate the desired dietary contrast. Crystalline methionine was added to the SBM-diets to achieve a balanced amino acid profile (NRC, 2011). The second factor aimed to create a contrast in dietary DON level. Control (CON) diets were aimed to be of free of the mycotoxin DON, which was achieved via the

Table 1
Ingredients composition, proximate and mycotoxin analysis of the experimental diets.

Experimental diets				
	Optimal quality		Sub-optimal quality	
Ingredients Inclusion (%)	CON-FM	DON-FM	CON-SBM	DON-SBM
Wheat 'clean'	40.00	-	40.00	-
Wheat 'contaminated'	-	40.00	-	40.00
LT fishmeal	37.01	37.01	15.13	15.13
Soybean meal (CP>45)	-	-	25.00	25.00
Fish oil	11.86	11.86	10.93	10.93
Blood meal	9.87	9.87	6.93	6.93
Monocalcium phosphate	0.13	0.13	0.70	0.70
Methionine	-	-	0.18	0.18
Choline	0.15	0.15	0.17	0.17
Premixes[a]	0.99	0.99	0.97	0.97
Analysed nutrient composition (%)[b]				
Dry Matter	94.6	94.9	92.4	94.3
Protein	44.7	44.4	38.7	38.9
Fat	17.0	17.0	14.6	14.8
Ash	7.3	7.1	5.7	5.8
Gross Energy (MJ/kg) DM	22.3	22.3	22.8	21.4
Mycotoxin concentration (µg/kg)[c]				
DON	-	1206	46	1329
DON-3-Glucoside	-	36	-	38
Enniatin A/A1	-	-	-	2
Enniatin B/B1	-	15	-	5
Mycophenolic Acid	-	-	34	80

[a] Commercial premix from Alltech Coppens to meet (NRC, 2011) requirements of rainbow trout.
[b] On dry matter basis, the symbol "-" means that the toxin was not present (0.00 µg/kg) or below the detection limit.
[c] In the main text, the rounded levels of DON-contaminated diets are mentioned: DON-FM: 1200, DON-SBM: 1300 µg/kg.

inclusion of 40% of a "clean" batch of wheat, confirmed free of DON and other toxins by liquid chromatography/tandem mass spectrometry (LC-MS/MS) at the Alltech 37 + mycotoxin laboratory (ISO/IEC 17025:2005 accredited) (Dunboyne, Ireland). DON-contaminated diets contained 40% "naturally contaminated" wheat, analysed for mycotoxin profiles and used in a previous study (Koletsi et al., 2022). Details on ingredient and analysed nutrient content of the four experimental diets (CON-FM, DON-FM, CON-SBM and DON-SBM) are presented in Table 1. Final feeds were analysed with LC-MS/MS to confirm the absence or low occurrence of DON in control, and high DON levels in contaminated diets (Table 1). Indeed, the CON-FM diet was free of DON and the CON-SBM diet had a minimal level of DON (46 μg/kg) and also mycophenolic acid (34 μg/kg). Both DON-contaminated diets (DON-FM and DON-SBM) had comparable DON levels of 1200 μg/kg and 1300 μg/kg, respectively, and also low concentrations of other toxins.

The experimental diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) as 2.5 mm extruded pellets. Fish were hand-fed twice daily. During the restrictive feeding period, the total amount of feed given per fish was equal for all treatments in order to have similar DON intake and similar dietary challenge between the experimental diets, following the 2 × 2 factorial design. Feeding equal amounts of feed and (therefore) DON should reveal the direct impact of the toxin on fish while excluding its potential effect on feed intake. The restrictive feeding was performed according to the metabolic body weight of the fish (12 $g/kg^{0.8}/d$) for six weeks. The daily amount of feed was increased throughout the restricted feeding period by predicting fish growth and weight, using the initial body weight and an expected FCR of 0.65. Halfway of restrictive feeding (week 3), fish were batch weighed for determination of biomass and FCR. The calculated average FCR of 0.84 was used to estimate the amount of feed until the end of the restrictive feeding. During the subsequent 2-week *ad libitum* feeding period, fish were fed to apparent satiation for one hour during each feeding event, in order to study the impact of DON and diet quality on feed intake. It was presumed that the fish were satiated and feeding stopped when uneaten pellets remained on the tank's bottom or floated on the water's surface for more than 10 min or when the feeding time of one hour was over. During both feeding periods, uneaten pellets were removed by siphoning and counted to measure feed intake.

2.3. Sampling and analyses

The sampling schedule and subsequent analyses were similar to those described for an earlier study (Koletsi et al., 2022). Briefly, for growth performance measurements, the biomass per tank was recorded at the start of the experiment and at the end of each feeding period, restrictive (week 6) and *ad libitum* (week 8). At week 6, five fish per tank were euthanised and stored at − 20 °C for body composition analysis. Fish carcass and feed samples were analysed by Nutricontrol (Veghel, The Netherlands). Dry matter was measured by drying at 103 °C until constant weight for 4 and 24 h, respectively (ISO 6496, 1999), crude protein based on nitrogen × 6.25 using the Kjeldahl method (ISO 5983, 2005), fat after an initial acid-hydrolysis step followed by a petroleum-diethyl ether extraction (ISO 6492, 1999), ash content after incineration at 550 °C for 4 h (ISO 5984, 2002) and gross energy content with the adiabatic bomb calorimeter method (ISO 9831, 1998).

For histopathological analysis, two sections of liver and a section from each segment of the GIT (pyloric caeca, midgut and hindgut) tissue from two fish per tank were collected at the end of week 1 and week 6 of restrictive feeding and at the end of the *ad libitum* feeding period (week 8). Samples were processed according to the histological procedures described by (Koletsi et al., 2022). Briefly, liver sections were stained separately with Periodic acid-Schiff's (PAS) reagent and with Haematoxylin and Eosin (H&E) and evaluated according to a previously developed scoring system (Koletsi et al., 2022). GIT sections were coloured with Alcian blue (pH 2.5) followed by Crossman, and pictures were imported in ImageJ software (version 1.53q) (Schindelin et al., 2012) to measure mucosal fold width, mucosal fold height, lamina propria width, enterocyte width, supranuclear vacuoles width (SNV) and goblet cell density.

Additionally, from the fish sampled for tissues (n = 2 per tank), total liver weight and total body length were recorded. During sampling, handling of the fish was avoided as much as possible while fish were euthanized by an overdose of benzocaine (dissolved in water at 0.5 ml/L). Samples were also collected from the initial population (before the start of the experiment and distribution to tanks, at time point zero) totalling 6 fish for tissue sampling and 20 fish for determining the initial body composition.

2.4. Calculations and statistical analysis

Growth performance parameters were calculated as follows: Weight gain (g) = FBW-IBW, FBW is the final body weight (g) and IBW the initial body weight (g); Growth (g/d) = weight gain/days; Specific growth rate (SGR, %/d) = ((ln FBW − ln IBW)/days) x 100; Feed conversion ratio (FCR) on DM basis = FI/weight gain, where FI is the feed intake defined as the average amount of feed (g) consumed by a fish, converted based to DM content of the feed (g/kg); Hepatosomatic index (HSI, %) = (liver weight/W) x 100; and Condition factor (K) = (W/L^3) x 100, where W is the individual FBW of the tissue sampled fish and L its body length (cm). Moreover, retained protein and energy and their retention efficiencies in rainbow trout whole body samples were calculated as follows: Retained protein (g/fish) = FBW x FPC - IBW x IPC, where FPC is the protein content (g) in the fish body and the end and IPC is the protein content (g) at the start; Protein retention efficiency (%) = (Retained protein/CPI) x 100, where CPI is the dietary protein intake (g/fish) calculated as = average FI of an individual x protein content in the feed. Similarly, for retained energy (MJ/fish) and energy retention efficiency (%); Retained energy = FBW x FEC - IBW x IEC, where FEC is the gross energy content (MJ) in the fish body and the end and IEC is the protein content (g) at the start; Energy retention efficiency (%) = (Retained energy/GEI) x 100, where GEI is the dietary gross energy intake (MJ/fish) calculated as = average FI of an individual x gross energy in the feed.

For the statistical analysis of growth performance and protein and energy retention, tanks (n = 12) were designated as the experimental units. The effect of dietary DON (CON versus DON) and diet composition (FM versus SBM) and their interaction was tested with a two-way ANOVA using the general linear model (GLM). Model residuals were tested for normality by using the Kolmogorov-Smirnov test and homogeneity of variance was determined by Levene's test. If interaction effects were significant, a Tukey's multiple comparison test was performed, with statistical significance being defined at a p-value ≤ 0.05. The histological parameters of the GIT were analysed separately for each segment (pyloric caeca, midgut and hindgut). Mixed-effects models were applied; a generalized linear model for the variables in the GIT and a multinomial logistic regression model for the continuous scores in the liver, with toxin (CON versus DON), diet (FM versus SBM), time (week 1, 6 and 8) and their interactions to be included in the model (n = 720, 60 per diet per time point), while fish was used as a random effect. The binomial (yes/no) liver data (nuclei pyknosis and pleomorphism, necrosis, haemorrhage, inflammation), were also analysed with mixed model using logistic regression with toxin, diet, time and their interactions as fixed effects and fish as a random effect in the model. The binomial outcomes were expressed as percentages (%) (n = 720, 60 per diet per time point). All data was statistically analysed in the IBM Statistical Package for the Social Sciences (SPSS) program (v 23.0; New York, NY, USA).

3. Results

3.1. Performance

During the entire experiment (eight weeks), no mortalities occurred, and no abnormal behaviour or difficulties in feed acceptance was observed.

3.1.1. Restrictive feeding period

During the restrictive feeding (6 weeks), both growth and FCR were affected by diet quality ($p \leq 0.001$) and by the presence of DON ($p \leq 0.05$). Growth of fish fed with SBM diets was 12% lower than growth of fish fed with FM diets, confirming that the experimental SBM-based diets could be considered sub-optimal. Growth of fish fed with diets containing DON had a 6% lower growth rate than fish fed with the CON diets (Table 2). These effects of diet quality and DON were additive, with an absence of an interaction effect ($p > 0.05$). In other words, the reduction in growth, or increase in FCR caused by DON were comparable for both optimal and sub-optimal quality diets.

Measurements of the hepatosomatic index (HSI) did not differ between treatments ($p > 0.05$), indicating there were no main effects on the liver caused by either diet quality, or DON. The condition factor (K), however, was different between diets because affected by diet quality; fish fed the SBM diets had a lower body condition score ($p \leq 0.05$; Table 2), confirming the experimental SBM-based diets could be considered sub-optimal. The treatments effects on retained protein and energy paralleled the pattern observed for growth. Both retained protein and retained energy were affected by DON and by diet (the two main effects), but there was no significant interaction (Table 2), meaning that the impact of DON on these parameters was similar in the groups fed an optimal- (FM) or a sub-optimal quality diet (SBM). Protein retention efficiency was affected only by DON; trout fed diets contaminated with DON had a 6% lower protein retention efficiency compared to trout fed the control diets ($p \leq 0.01$). The only performance indicator affected by the interaction effect of diet quality and DON ($p \leq 0.05$) was energy retention efficiency; exposure of fish to DON reduced the energy retention efficiency in the FM diets but not in the SBM diets (Table 2). These outcomes indicate that a sub-optimal diet such as SBM does not necessarily aggravate DON effects on performance.

3.1.2. Ad libitum feeding period

During the 2 weeks of *ad libitum* feeding, daily feed intake was not affected by diet quality, nor by the presence of DON (Table 3), and with an average of 1.8–1.9 g/fish/day, feed intake was highly similar among treatments. Although over this relatively short feeding period diet quality did not affect growth, the *ad libitum* feeding did result in DON affecting growth ($p \leq 0.05$); trout fed with DON diets had an 11% lower growth rate than trout fed with CON diets. FCR was affected by both the effects of DON ($p \leq 0.001$) and diet quality ($p \leq 0.001$). FCR was 9% higher in DON compared to CON diets and 9% higher in SBM compared to FM diets, indicating that the two factors (DON and diet quality) contribute equally to a poorer feed efficiency in trout. Both growth and FCR were unaffected by the interaction effect ($p > 0.05$).

HSI was reduced by the presence of DON in the diet ($p \leq 0.05$), possibly indicative of DON negatively affecting liver health, but was unaffected by the diet quality ($p > 0.05$). HSI was equal for both FM diets, whilst the addition of DON to the SBM diet numerically reduced HSI (DON-SBM<CON-SBM). Though the interaction effect between DON and diet quality was not statistically significant, the outcome still implies that DON effects on the liver (HSI) were more obvious against the background of a less optimal diet. The condition factor (K), however, was different between diets and was thus affected by diet quality; fish fed the SBM diets had a lower body condition score ($p \leq 0.05$; Table 2), confirming the experimental SBM-based diets could be considered sub-optimal. Finally, there was no effect of DON on condition factor, regardless of whether fish were fed a FM- or SBM-based diet ($p > 0.05$).

3.2. Health

3.2.1. Histopathological assessment of the gastrointestinal tract (GIT)

Qualitative assessment of the histological pictures from the GIT did not reveal obvious clinical alterations. Representative pictures from intestinal folds after *ad libitum* exposure (week 8) are presented in Fig. 1. Further examples during and after restrictive exposure (week 1 and week 6) are also available (tile scans, Figs. S1, S2). Diet quality (i.e., FM versus SBM diets) seemed to cause some histological alterations in the intestine; a slight reduction of the width and a more irregular appearance of the zone with supranuclear vacuoles (SNV) was visible especially in the hindgut (Fig. 1). Feeding diets with DON did not cause obvious histological changes in the intestine.

Quantitative assessment should allow for detection of statistically significant alterations in the GIT (pyloric caeca, midgut and hindgut) induced by the imposed factors, such as DON, diet and time (Table S1). No significant histological effects of DON intake were observed in the hindgut or the pyloric caeca within the GIT. However, DON-induced alterations were detected in the midgut area, where a main effect of DON over time was present on the width of the area with supranuclear vacuoles (SNV) ($p \leq 0.05$). This suggests that DON may have an effect on the GIT, detected as reduced zone with SNV ($p \leq 0.05$; CON diets: 44.8 μm versus DON diets: 41.0 μm) in the midgut, with the effect being dependent upon the duration of the feeding period. Interaction effects between DON and diet were present and significant for several histopathological parameters measured in the midgut. Both, mucosal fold width and enterocyte width, were reduced by DON against the background of a sub-optimal SBM-based diet, but not FM-based diet

Table 2

Effects of dietary DON, diet quality (FM versus SBM) and their interaction on the performance of rainbow trout fed the experimental diets (CON-FM diet; DON= 0 μg/kg, DON-FM; DON=1200 μg/kg, CON-SBM; DON=46 μg/kg and DON-SBM; DON=1300 μg/kg) during a 6-week restrictive feeding period.

	Experimental diets					p-value		
Growth parameters	CON-FM	DON-FM	CON-SBM	DON-SBM	SEM	DON	DIET	DON*DIET
Initial BW (g)	9.91	9.99	9.97	9.90	0.122	NS	NS	NS
Final BW (g)	40.6	39.2	37.1	35.6	0.50	*	* **	NS
Growth (g/d)	0.77	0.73	0.68	0.64	0.012	*	* **	NS
SGR (%BW/d)	3.53	3.42	3.28	3.20	0.041	*	* **	NS
FCR	0.70	0.73	0.79	0.83	0.012	*	* **	NS
HSI (%)	2.26	2.30	2.17	2.00	0.23	NS	NS	NS
Condition factor (K)	1.23	1.18	1.15	1.14	0.022	NS	*	NS
Retained protein (g/fish)	4.80	4.49	4.16	3.90	0.078	* *	* **	NS
Protein retention efficiency (%)	50.3	47.5	49.9	46.8	0.8	* *	NS	NS
Retained energy (MJ/fish)	0.239	0.222	0.202	0.194	0.004	* *	* **	NS
Energy retention efficiency (%)	50.3^{a}	46.6^{b}	41.4^{c}	42.5^{c}	0.74	NS	* **	*

FM: fishmeal-based diet, SBM: soybean meal-based diet, BW: body weight, SGR: specific growth rate, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, NS: not significant, * ** : $p \leq 0.001$, * *:$p \leq 0.01$, * : $p \leq 0.05$. Treatments lacking a common letter are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison test.

Table 3
Effects of dietary DON and diet quality (FM versus SBM) on the performance of rainbow trout fed the experimental diets (CON-FM diet; DON=0 μg/kg, DON-FM; DON=1200 μg/kg, CON-SBM; DON=46 μg/kg and DON-SBM; DON=1300 μg/kg) during a 2-week *ad libitum* feeding period.

	Experimental diets					**p-value**		
Growth parameters	CON-FM	DON-FM	CON-SBM	DON-SBM	SEM	DON	DIET	DON*DIET
Final BW (g)	73.7	70.2	68.6	63.1	1.94	*	*	NS
Growth (g/d)	2.23	2.03	2.07	1.82	0.092	*	NS	NS
SGR (%BW/d)	4.03	3.79	4.02	3.79	0.110	NS	NS	NS
Average daily feed intake (g/fish/day)	1.88	1.84	1.92	1.83	0.062	NS	NS	NS
FCR	0.80	0.86	0.86	0.95	0.015	* **	* **	NS
HSI (%)	2.3	2.3	2.7	2.0	0.22	*	NS	NS
Condition factor (K)	1.23	1.18	1.15	1.14	0.022	NS	*	NS

FM: fishmeal-based diet, SBM: soybean meal-based diet, BW: body weight, SGR: specific growth rate, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, NS: not significant, * ** : $p \leq 0.001$, * *:$p \leq 0.01$, * : $p \leq 0.05$.

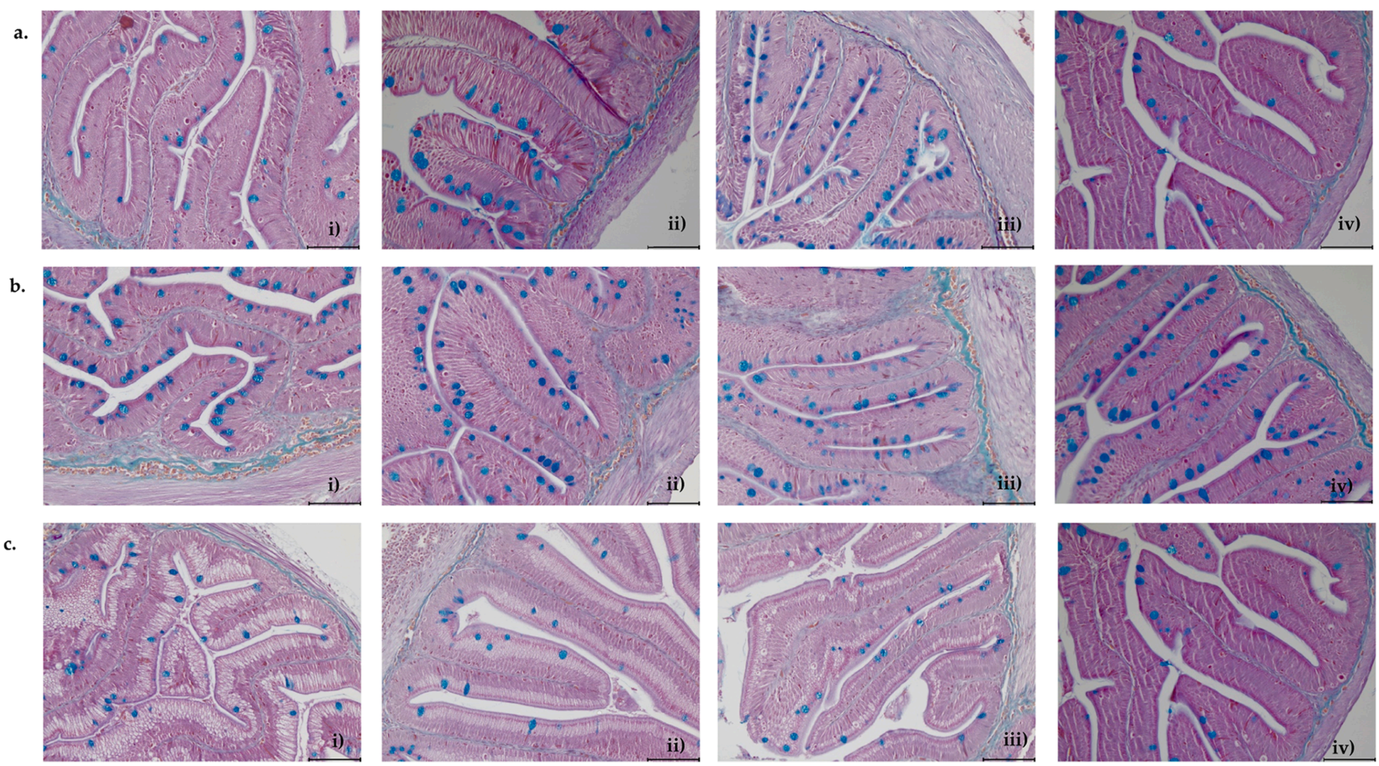

Fig. 1. Representative examples of histological sections of the intestinal folds in **a)** pyloric caeca **b)** midgut and **c)** hindgut of rainbow trout fed: **i)** a control fishmeal-based diet without mycotoxins (CON-FM), **ii)** a fish meal based diet with DON (DON-FM), **iii) a** control soybean meal-based diet without mycotoxins (CON-SBM) and iv) a soybean meal-based diet with DON (DON-SBM) *ad libitum* for 2 weeks. Staining: Alcian blue-Crossman; Magnification: x 20; Black scale bar = 100 μm.

($p \leq 0.05$; Fig. 2). Also, the number of goblet cells in the midgut was highest in fish fed a CON-SBM diet, and intermediate in fish fed DON, independent of diet quality ($p \leq 0.05$; Fig. 2).

The main effects of diet quality on the different histopathological parameters measured in the GIT are presented in Table 4 and shortly summarized below. Mucosal fold width was reduced in fish fed SBM diets compared to fish fed FM diets, at least in the pyloric caeca and midgut ($p \leq 0.05$). Mucosal fold height was reduced in fish fed SBM diets compared to fish fed FM diets, but only in the midgut ($p \leq 0.05$). Lamina propria width was unaffected by diet quality in all gut segments ($p > 0.05$). As previously highlighted, enterocyte width was unaffected by diet quality, with the exception of the interaction effect between diet quality and DON in the midgut. Moreover, the width of the SNV layer inside the enterocytes was reduced by replacing FM by SBM, in all gut segments ($p \leq 0.05$). Goblet cell density, i.e., the number of goblet cells in the pyloric caeca and hindgut were not influenced by diet (Table 4). Some of the histopathological parameters were affected by time (Table S1), but are not discussed here since for almost all parameters investigated, 2 and 3-way interaction effects with time were not significant.

3.2.2. *Histopathological assessment of the liver*

Qualitative analysis of the livers for histopathological signs indicated the occasional presence of necrotic areas which seemed inconsistent with treatment, but rather varied greatly within each of the treatment groups; necrosis was observed in groups fed DON, but also in control groups not fed with DON (Fig. 3), and in groups fed both diet qualities (Fig. S3). In line with this high variation within groups, subsequent quantitative analysis of the livers for histopathological signs did not reveal significant effects of DON, diet quality, or time (Table S2). Notably, although necrotic areas may have been present to some extent in all groups, including fish fed CON diets, mean necrosis score was generally low (<0.7), as was the presence of haemorrhages and inflammation in all treatment groups during the whole experiment. The only exception was the time main effect ($p \leq 0.01$) and the significant 3-way interaction effect of DON, diet quality and time on the lipid

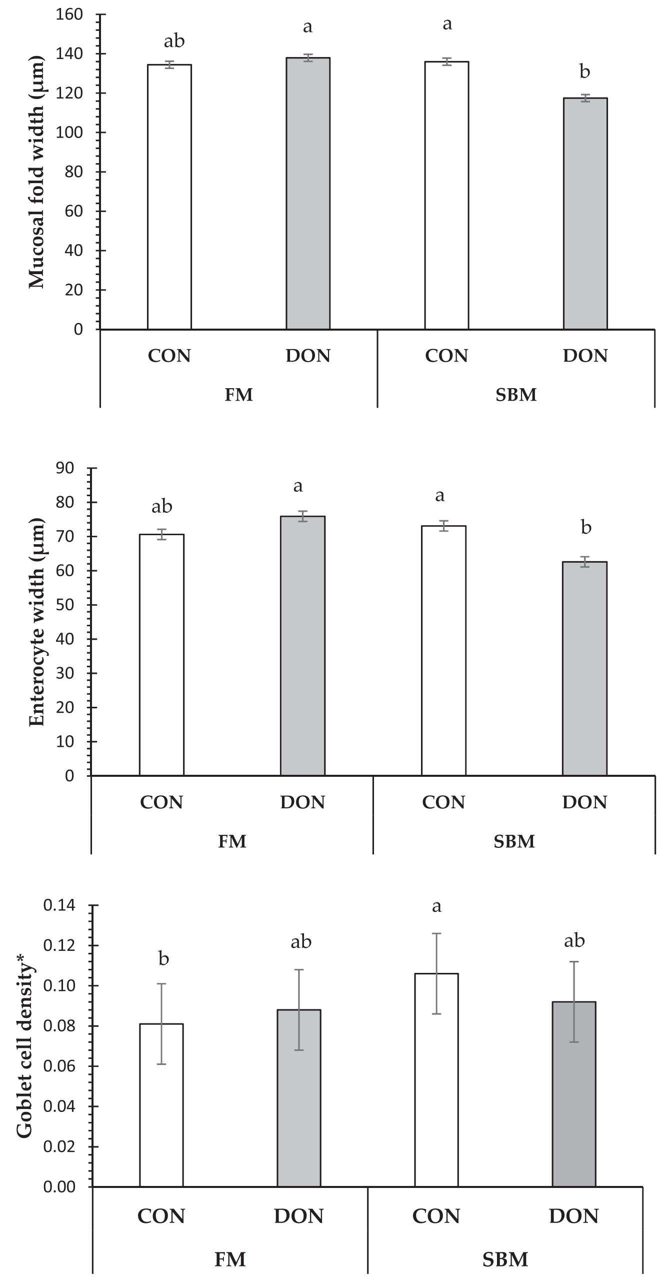

Fig. 2. Interaction effects between dietary DON (CON versus DON) and diet quality (FM versus SBM) on mucosal fold width ($p \leq 0.05$), enterocyte width ($p \leq 0.05$) and goblet cell density ($p \leq 0.05$) in the midgut of rainbow trout fed the experimental diets: CON-FM, DON-FM, CON-SBM and DON-SBM restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* exposure for 15 days (week 8). *Goblet cell density was calculated as the number of cells per µm fold height. Error bars indicate standard error of means. Treatments lacking a common letter are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison test.

vacuolisation ($p \leq 0.01$); within the FM group, lipid vacuolization was increased in the DON diets compared to the CON diets by week 8.

4. Discussion

Previously, our study in rainbow trout indicated that dietary exposure to DON can reduce growth and have time-dependent effects on fish health in terms of liver damage, and may also negatively impact the integrity of the intestinal barrier (Koletsi et al., 2022). While we observed negative effects on performance and health induced by DON, this previous study was performed with an optimal-quality marine--based diet. It is possible that a sub-optimal plant-based diet could further worsen the observed negative effects of DON. To gain insight in such possible interactions between DON and diet composition, the present study investigated the effects of DON by studying fish performance and health in experimental treatments fed a fishmeal (FM)-based diet versus fish fed a sub-optimal diet based on soybean meal (SBM). The data from our experiment indicate that the impact of DON on growth and liver health did not differ between the FM- and SBM-based diets. This was shown by the absence of an interaction effect between DON and diet quality, suggesting that combined effects of DON and diet composition were additive. Although during the *ad libitum* feeding period, numerically the effect of DON on FCR was larger in fish fed the SBM diets compared to the FM diets. This is in line with the hypothesis of a poorer expected performance in trout fed the sub-optimal quality diet. The data from our experiment also indicate that the impact of DON on intestinal health did differ between the FM- and SBM-based diets. The interaction effect between DON and diet quality was observed for particular histopathological parameters, for example, dietary DON contamination aggravated a number of SBM-induced enteritis symptoms in the midgut area of the gastrointestinal tract. Before discussing the interaction between DON and diet quality, first the main effects of DON and diet quality on trout performance and health will be discussed.

Table 4
Main effect of dietary quality (FM versus SBM) on histological parameters in pyloric caeca, midgut and hindgut of rainbow trout fed restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* for 15 days (week 8) the experimental diets; CON-FM diet (DON= 0 µg/kg), DON-FM (DON=1200 µg/kg), CON-SBM (DON= 46 µg/kg) and DON-SBM (DON= 1300 µg/kg).

	Dietary Composition			
	FM	SBM	SEM[a]	p-value
Mucosal fold width (µm)				
Pyloric	155.7	144.0	3.60	*
Midgut	137.1	127.2	3.34	*
Hindgut	150.7	141.5	4.23	NS
Mucosal fold height (µm)				
Pyloric	436.5	401.1	20.85	NS
Midgut	372.9	336.4	10.55	*
Hindgut	383.6	375.2	15.22	NS
Lamina propria width (µm)				
Pyloric	16.16	16.00	0.64	NS
Midgut	17.9	17.5	0.60	NS
Hindgut	17.7	17.2	0.65	NS
Enterocyte width (µm)				
Pyloric	85.5	78.4	2.42	*
Midgut	73.7	68.1	2.35	NS
Hindgut	65.0	65.5	2.44	NS
Supranuclear vacuole (SNV) width (µm)				
Pyloric	53.0	48.8	1.50	*
Midgut	44.9	40.8	1.14	*
Hindgut	67.0	57.8	2.95	*
Goblet cell density[b]				
Pyloric	0.038	0.043	0.003	NS
Midgut	0.085	0.100	0.004	* *
Hindgut	0.054	0.049	0.004	NS

[a] Pooled standard error of means: SEM (total cases n = 720, included cases in pyloric: n = 695, midgut: n = 600, hindgut: n = 550)
[b] Calculated per µm fold height, Not significant: NS, $p \leq 0.05$: * , $p \leq 0.01$: *

4.1. The effects of DON on performance metrics

With regard to performance, during both restrictive and *ad libitum* feeding in our study, exposure to industrially-relevant DON levels (1200–1300 µg/kg) reduced weight gain and feed efficiency of rainbow trout. The current results on a reduced performance during satiation feeding have been reported in various studies in rainbow trout

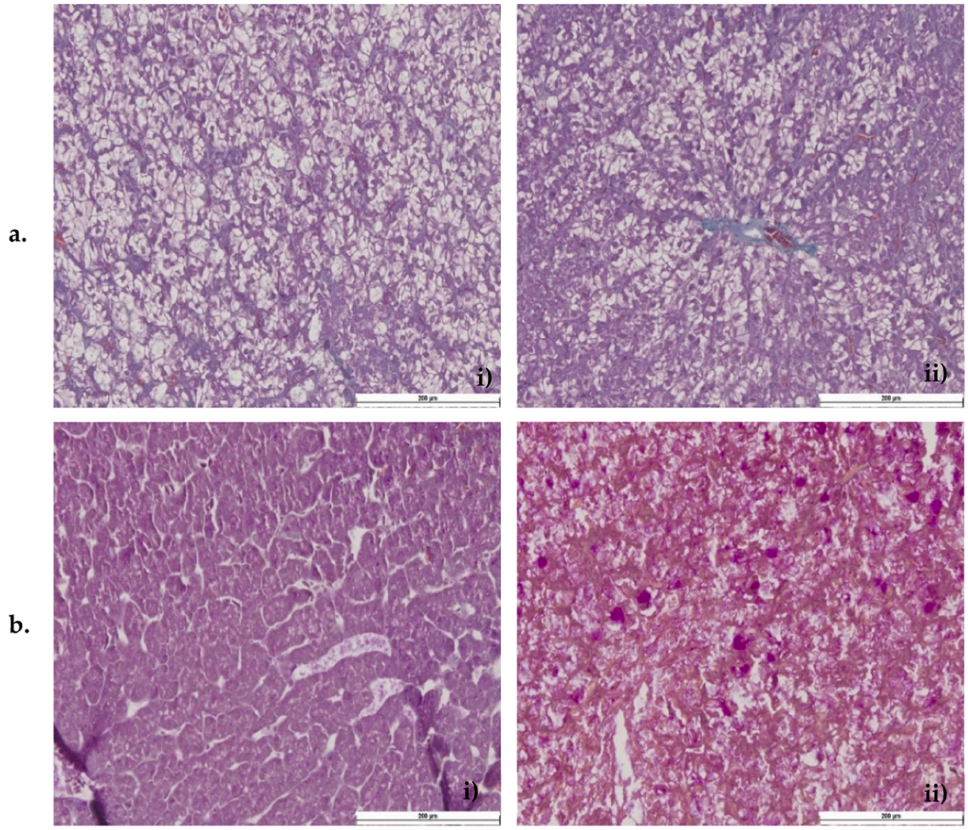

Fig. 3. Representative examples of histological sections of the liver from rainbow trout fed **i)** a control fishmeal-based diet without mycotoxins (CON-FM) and **ii**) a control fishmeal-based diet with DON (DON-FM). The first row **(a)** shows representative pictures per diet without pathological indication and the second row **b)** examples of livers with necrotic areas. Staining: PAS-Crossman; Magnification: x 20; White *s*cale bar = 200 *μm*.

(Gonçalves et al., 2019; Gonçalves et al., 2018; Hooft and Bureau, 2017; Hooft et al., 2011; Hooft et al., 2019a; Hooft et al., 2019b; Ryerse et al., 2015). In slight contrast to our previous study (Koletsi et al., 2022), which used similar DON levels, growth and FCR were negatively affected in this experiment. Yet, in line with our previous observations (Koletsi et al., 2022), again, impairment of performance during the restrictive feeding period was mirrored by suppressed protein and energy gain. Together, our studies suggest that exposure of rainbow trout to levels of DON that are of practical relevance for aquaculture has a direct effect on growth, possibly through inhibition of protein synthesis. Furthermore, during the restrictive feeding period, the estimated daily intake (EDI, μg/g BW/day) of DON in rainbow trout was 0.028 (DON-FM) and 0.033 (DON-SBM), comparable with the EDI calculated for the naturally DON-contaminated diet (1300 μg/kg), 0.033 (Koletsi et al., 2022). Comparing our earlier study with the present study shows that despite similar exposure levels, the effects of DON can vary, which was also the case for histopathological effects on the liver.

4.2. The effects of DON on health metrics

With regard to health, in the present study, DON exposure did not induce histopathological changes in the liver, while using similar levels of DON in our previous study did affect liver health (Koletsi et al., 2022). In most DON studies in trout, the liver is the major organ of study due to its sensitivity to DON (Hooft and Bureau, 2021; Koletsi et al., 2022). Here, even after the *ad libitum* feeding period, hepatic damage was not obvious, despite a relatively high EDI for DON, estimated at 0.040 μg/g BW/day (DON-FM diet), or 0.049 μg/g BW/day (DON-SBM diet). In our previous study, although the EDI was comparable (0.044 μg/g BW/day DON diet) to the present study, we did observe DON effects on the liver after *ad libitum* exposure to DON (Koletsi et al., 2022). In general, there is high variability in the responses to DON among studies with the same design. The variability might be related to the different life histories of fish batches, the mycotoxin profile in the naturally contaminated ingredients or even the statistical power of the study or duration of the exposure. Similar to our previous study (Koletsi et al., 2022), DON did not affect feed intake during *ad libitum* exposure. These observations are opposite to the reduced feed intake even at practical relevant DON levels (1000–1500 μg/kg) found in a meta-analysis on trout (Koletsi et al., 2021). The absence of a reduction in feed intake by DON may be due to the short duration of the *ad libitum* feeding period. Another reason might be that trout were already adapted to DON exposure prior to the *ad libitum* period since fish were already fed the DON diets during the six weeks of the restrictive feeding period. Studies in pigs have shown early effects of DON on feed intake from the first 7 days (Serviento et al., 2018) to 4 weeks (Wellington et al., 2020) of exposure, whilst later the animals become adapted to the contaminated diets.

4.3. The impact of diet quality

The dietary composition significantly affected the performance of rainbow trout; 25% of SBM resulted in reduced growth, feed efficiency, protein and energy gain, confirming its challenging nature for salmonids. A key factor for the suppressed performance might be related to the known antinutritional factors of SBM and the lower protein and lipid content to a lesser extent. Regarding GIT, a sub-optimal diet with 25% SBM inclusion aimed to have a mild response, although it did not affect histopathological parameters linked to inflammation. There was no widening of lamina propria and infiltration of inflammatory cells as has previously been described for salmon (Krogdahl et al., 2003; van den Ingh et al., 1996). Our findings are in contrast to observations in rainbow trout where 40% SBM inclusion caused granulomatous enteritis

(Mosberian-Tanha et al., 2018). Our histopathological assessment did however detect a reduced gut mucosal fold width and height, reduced SNV width and an increased goblet cell density, all of which have been previously described as being indicative of enteritis in Atlantic salmon (van den Ingh et al., 1991). Surprisingly, most of these changes occurred in the midgut and not in the hindgut in the current study. The hindgut is normally the affected part of the intestine by SBM in salmonids (van den Ingh et al., 1991). A recent meta-analysis in salmon (Agboola et al., 2022) showed that the most affected variable in the assessments of SBM enteritis in salmon is the loss of SNV, which was the only parameter that was affected in all intestinal segments of our SBM-treated trout, including the hindgut. The lack of enteritis severity in the hindgut might be related to the 25% inclusion level of SBM, which is lower than 30% when enteritis seems to develop in trout (Refstie et al., 2000). However, the severity is mostly governed by the source of SBM instead of the inclusion level (Agboola et al., 2022; URÁN et al., 2009). Indeed, the severity of SBM-induced enteritis seems to have declined over the years (after 2014) due to improved diet formulations, processing methods for SBM that minimize anti-nutritional factors, and the genetic selection of fish that are adapted to plant-based diets (Agboola et al., 2022).

4.4. Interaction of DON and diet quality

We did not find that diet composition influences the impact of DON on trout, on either the growth performance or health parameters measured. Although this suggests that in rainbow trout DON-induced effects on performance and health may be such that they overrule effects induced/modulated by diet, it could be that the current experimental set-up relied on a sample size of insufficient power to detect interaction effects. Yet, there are no clear indications for a limited power of this study, for example, during the *ad libitum* feeding period, the reduction in growth at both FM and SBM diets was numerically equal. It does not seem likely that the absence of an interaction effect would be due to low DON exposure in the current study (1200–1300 μg/kg). Although often higher DON doses are tested in trout experiments along with slightly longer periods of *ad libitum* exposure of up to eight weeks (Gonçalves et al., 2018; Hooft et al., 2011; Hooft et al., 2019a), our data showed that DON exposure did affect growth, FCR, protein and energy gain, which suggests the applied DON levels may have been relatively mild but sufficient to induce changes. In fact, at (very) high DON levels an interaction between DON and diet composition usually is not present, as indicated by a meta-analysis for trout (Koletsi et al., 2021), also because feed intake declines exponentially with increasing DON levels. Indeed, it is most likely that interaction effects with diet quality would be most easily detected against a backdrop of mild effects induced by DON, as in the current study. It is not unlikely that the absence of a clear interaction effect with diet quality could have been due to a relatively small contrast between the experimental diets based on FM, or SBM. It could be that the level and/or type of SBM included in the diet was not challenging enough to affect performance and/or health to a great extent. Indeed, in the current study, 25% inclusion of SBM only led to relatively small changes in the gastrointestinal tract of rainbow trout. Future studies addressing the influence of dietary composition on effects of DON on performance, liver and gastrointestinal tract health of rainbow trout could consider designing experimental diets with a greater contrast in diet quality.

Last but not least, one could hypothesize that interaction effects would be absent because dietary composition (i.e. effects of SBM) affects/targets different parts of the GIT than does exposure to DON. DON is known to be quickly taken up in the pyloric caeca region of the intestine and afterwards is distributed to organs including the liver (Bernhoft et al., 2017). In contrast, SBM-induced enteritis in salmon is mainly present in the distal and not in the upper part of the gastrointestinal tract (van den Ingh et al., 1991). Maybe arguing against this hypothesis are our observations that some histopathological parameters in the GIT (mucosal fold width; enterocyte width) did show an interaction effect between DON and diet composition. Changes in these parameters induced by SBM inclusion in the diets were largest when DON was also present in the diets, suggesting that DON may have enhanced SBM-induced enteritis, although only observed in the midgut. A complete understanding of the potential role of the midgut in developing SBM-induced enteritis and interaction effects with DON in rainbow trout would require further research on the use of plant ingredients in salmonid diets. To date, dietary SBM-induced effects on rainbow trout have only been co-evaluated with other types of challenges common to aquaculture practices, i.e. hypoxia (Mosberian-Tanha et al., 2018) and salinity (Nordrum et al., 2000), but not exposure to DON or other mycotoxins, making our study unique.

5. Conclusion

This study did not confirm that diet composition influences the impact of DON in rainbow trout, based on metrics of growth performance and liver histology. Regarding intestinal histology, the present research found that DON can alter SBM-induced enteritis symptoms but only in the midgut. Mucosal fold width and enterocyte width were reduced by DON only in the SBM-treated trout. In future studies, it might be worthwhile to further explore the DON exposure and SBM challenge in the midgut by employing an in vitro epithelial barrier model (e.g. RTgutGC cell line from rainbow trout). This approach might allow us to zoom into the cellular level and understand further unknown pathological changes in the midgut of rainbow trout. Overall, our findings show that DON levels ≥ 1200 μg/kg have a negative impact on trout growth performance regardless of the dietary composition; the effects were similar in trout fed FM- and SBM-based diet. This information is relevant for the industry, perhaps leading to a more flexible formulation of aquafeeds allowing higher inclusion of alternative ingredients other than fishmeal without aggravating mycotoxins effects.

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CRediT authorship contribution statement

Conceptualization: P.K., J.S.; Methodology: P.K., J.S., E.A.M.G; Software: P.K., E.A.M.G.; Validation: P.K., J.S., E.A.M.G.; Formal analysis: P.K., E.A.M.G.; Investigation: P.K., J.S.; Resources: G.F.W. and P.L.; Data Curation: P.K., J.S.; Writing — original draft: P.K; Writing — review and editing: P.K., J.S., E.A.M.G., G.F.W., P.L.; Visualization: P.K.; Supervision: J.S., G.F.W. and P.L; Project administration: P.K., G.F.W. and P.L.; Funding acquisition: G.F.W. and P.L. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paraskevi Koletsi reports financial support was provided by Alltech. Paraskevi Koletsi reports a relationship with Alltech that includes: funding grants.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101740.

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