Fish and Shellfish Immunology 86 (2019) 1106–1113

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Gut health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black soldier fly (*Hermetia illucens*) larvae meal

Yanxian Li[a,*], Trond M. Kortner[a], Elvis M. Chikwati[a], Hetron Mweemba Munang'andu[a], Erik-Jan Lock[b], Åshild Krogdahl[a]

[a] Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), P.O. Box 8146 Dep, NO-0033, Oslo, Norway
[b] Institute of Marine Research, P.O. Box 1870 Nordnes, 5817, Bergen, Norway

ARTICLE INFO

Keywords:
Black soldier fly larvae
Atlantic salmon
Gut health
Immune response
Antibody
Vaccination

ABSTRACT

Limited availability of sustainable feed ingredients is a serious concern in salmon aquaculture. Insects may become an important, sustainable resource for expanding the raw material repertoire. Herein, we present data from an 8-week feeding trial with pre-smolt Atlantic salmon (initial body weight 49 ± 1.5 g) fed either a reference diet containing fish meal, soy protein concentrate and wheat gluten as protein sources, or a test diet wherein 85% of the protein was supplied by black soldier fly larvae meal. Possible diet effect on the systemic immune response was evaluated by measuring plasma antibody titers after vaccination against infectious pancreatic necrosis virus (IPNV). The gut health of fish was evaluated using endpoints including organ and tissue indices, histopathological parameters and gene expression. Both diets induced the same level of antibody responses against IPNV. In fish fed the reference diet, the histological examination of the pyloric caeca mucosa showed clear hyper-vacuolization suggestive of lipid accumulation in enterocytes, whereas this was less pronounced in the insect meal fed fish. Expression of genes relevant to lipid metabolism confirmed these histological findings. Immune and barrier-function gene expression profiles were both generally not affected by diet. However, the fish fed insect meal showed increased expression of genes indicative of stress response, immune tolerance and increased detoxification activity. In summary, our results showed no indications that dietary inclusion of insect meal affected the gut health of Atlantic salmon negatively. The insect meal based diet seemed to reduce excessive lipid deposition in the pyloric caeca and stimulate xenobiotic metabolism.

1. Introduction

The world's wild fish catches seem to have reached their limit, whereas fishmeal and fish oil demands continue to grow, mostly due to the rapid growth in aquaculture. Therefore, marine ingredients in salmon diets have been gradually replaced by plant ingredients, decreasing from ~90% in 1990 to ~35% in 2012 [1]. Of the plant-based protein sources, 58% was from soy protein concentrate (SPC) followed by sunflower expeller (16%), wheat gluten (16%), fava beans (5%) and other marginally used plant proteins. However, some of these plant-based ingredients cause proliferative or inflammatory conditions in the intestinal mucosa as well as changes in host immune responses and gut enzymatic profiles, while others have been reported to increase fish susceptibility to various diseases [2–5]. Although the future availability of SPC is guaranteed in the short-term [6], there is a need for new nutrient sources in salmon aquaculture to cope with the increasing demand. Moreover, as the world population is projected to reach 9 billion in 2050, global food production must maximize nutritional output for human consumption while minimizing the input of resources [1]. Salmon feed producers need to reduce their dependency on terrestrial plant products that may be used directly for human consumption. Hence, several novel feed ingredients have emerged as promising candidates such as bacterial meal (*Methylococcus capsulatus*) [6], yeasts [7] and insects [8].

Insects are promising alternative protein sources as they possess an outstanding capacity to upgrade low-quality organic material, require minimal water and cultivable land, and emit little greenhouse gases [9]. Among species with the greatest potential for large-scale feed production are black soldier fly (BSF; *Hermetia illucens*), yellow mealworm (*Tenebrio molitor*), silkworm (*Bombyx mori*) and common housefly (*Musca domestica*) [10]. Black soldier fly has been produced at industrial scale in Europe due to its exceptionally good nutritional value

* Corresponding author.
E-mail address: yanxian.li@nmbu.no (Y. Li).

https://doi.org/10.1016/j.fsi.2018.12.057
Received 4 September 2018; Received in revised form 26 November 2018; Accepted 23 December 2018
Available online 24 December 2018
1050-4648/

and suitability for massive production [11–14]. On a dry matter basis, BSF larvae contain about 42% protein and 35% lipid [12]. In a survey of the nutritional composition of various insects belonging to the order Diptera, Orthoptera and Coleoptera, Barroso et al. found that BSF prepupae possessed an essential amino acid profile closest to fishmeal [14]. While the amino acid profile of insects is genetically determined and stable, the fat level and fatty acid profile are diet-dependent [13]. The potential of BSF larvae as a feed ingredient has been evaluated in several carnivorous and omnivorous fish species [13,15–25]. The optimal inclusion level of BSF larvae meal in the diet seems to vary considerably among fish species. For Atlantic salmon, results by Lock et al. have indicated that all fish meal could be replaced by BSF larvae meal without affecting the salmon growth performance or sensory quality [25]. Together, these results suggest that BSF is a realistic protein source for the salmon aquaculture.

A prerequisite for introducing a new ingredient into fish feed is convincing evidence that it will not compromise fish health. Intestinal inflammation and lipid malabsorption are two frequently observed gut health problems associated with increased use of plant-based salmon feed [4,26–29]. How feed ingredients of insect-origin may affect gut health and function is one of the main research questions that must be answered before commencing on full-scale commercial production. One of the immunological reactive compounds in insect meals is chitin, an essential component of the insect cuticle also found in bacterial and fungal cell walls. Chitin polymers (40–70 μm) act as pathogen associated molecular patterns (PAMPs) that bind pattern recognition receptors (PRRs) on various antigen presenting cells stimulating the production of various cytokines and immune mediators [30]. It may also interfere with protein and lipid digestion. However, positive effects such as growth promotion and immune-stimulation in fish have been reported [31,32]. Although impacts of BSF dietary inclusion on growth performance, sensory, feed conversion ratio and several other factors have been investigated in different fish species [13,15,20,33], its effect on gut health, function and host immune response has not been elucidated. Hence, the objectives of this study with Atlantic salmon are to: (i) evaluate the morphological and organosomatic changes induced by BSF in the gut, (ii) profile host immune gene expression evoked by BSF, (iii) and to evaluate the impact of BSF on antibody responses induced by parenteral vaccination.

2. Materials and methods

2.1. Diets and fish husbandry

An 8-week feeding trial was conducted at the Cargill AquaNutrition (former EWOS Innovation) experimental facility at Dirdal, Norway, in accordance with laws regulating the experimentation with live animals in Norway. A total of 800 mixed-gender pre-smolt Atlantic salmon with a mean initial body weight of 49 g (1.5 g SEM) were randomly assigned into 8 fiberglass tanks (450 L, 100 fish per tank) supplied with running freshwater. Quadruplicate tanks of fish were fed either a reference diet (REF) containing fish meal, soy protein concentrate and wheat gluten as protein sources, or an insect meal based diet (IM) wherein 85% of the protein was supplied by black soldier fly larvae meal, replacing the bulk of fish meal and soy protein (Table 1). The fish were fed continuously by automatic disk feeders under a photoperiod regimen of 24 h daylight. During the feeding trial, water temperature stabilized around 13.7 °C. Further details on insect meal and diet composition (amino acids, fatty acids and minerals) were reported elsewhere [34].

2.2. Sample collection

At the termination of the feeding trial, fish were randomly taken from the tanks, anesthetized with tricaine methanesulfonate (MS222) (80 mg/L; Pharmaq AS, Oslo, Norway) and euthanized by a sharp blow to the head before tissue sampling. The body weight was recorded for all the fish sampled. Subsequently, the whole digestive tract was dissected from three fish per tank, cleaned of attached adipose tissue and opened longitudinally. Only fish with chyme present along the whole intestine were sampled to ensure exposure to the diets until the point of sampling. The chyme was removed using a spatula. The emptied intestine was divided into proximal (PI), mid (MI) and distal (DI) segments and weighed respectively. The gut tissue was rinsed in phosphate buffered saline (PBS) three times to remove traces of remaining chyme and cut into pieces for RNA extraction (preserved in RNAlater solution, incubated at 4 °C for 24 h and stored at −20 °C) and histological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage).

Table 1
Formulation of the experimental diets*.

Ingredients (g/100 g)	REF	IM
Fishmeal LT94	35.0	6.0
Insect meal	0	60.0
Soy protein concentrate	29.6	5.0
Wheat gluten	14.3	14.4
Fish oil	4.6	6.9
Rapeseed Oil	12.0	4.8
Vitamin & mineral premix	0.3	0.3
Yttrium	0.2	0.2
Miscellaneous	4.0	2.4
Chemical composition		
DM (%)	94	96
Crude lipid (%)	18	22
Crude protein (%)	47	44
Carbohydrates (%)	11	12
Ash (%)	8	7
Gross energy (MJ Kg^{-1} DM)	22	23
TBARS (nmol g^{-1})	7	17

*REF, reference diet; IM, insect meal diet; DM, dry matter; TBARS, Thiobarbituric acid reactive substances.

2.3. Vaccination and ELISA

To investigate possible diet effect on the systemic immune response, another 20 fish were randomly selected from each tank and intraperitoneally injected with 0.1 mL of inactivated whole viral vaccine based on the highly immunogenic strain of infectious pancreatic necrosis virus (IPNV) [35]. Due to constraints on the availability of experimental facilities, the quadruplicate tanks per diet group were reduced to duplicate tanks per diet with 40 fish each. The fish were fed the same diets for another six hundred degree days. Thereafter, blood samples were taken from the caudal vein of 20 fish per tank using heparinized syringes equipped with 22 G needles (35 IU heparin per mL; S-Monovette®, SARSTEDT, Germany) and placed on ice before plasma collection. Plasma was collected after centrifugation at 2000 g for 10 min (4 °C) and snap-frozen in liquid N_2 for the determination of antibody level against IPNV.

The vaccine was prepared after culturing IPNV in Asian Grouper-strain K cells [36] at a concentration of 1×10^9 $TCID_{50}$/mL as previously standardized [37,38]. The virus suspension was inactivated using 0.5% formaldehyde using a magnetic stirrer for 48 h followed by dialysis for 48 h. The vaccine was prepared as a water-in-oil (w/o) formulation using the ISA 763 VG (SEPPIC, France) adjuvant following manufacturer's recommendation. Emulsification of the viral antigen with adjuvant was done as previously described [35].

The enzyme linked immunosorbent assay (ELISA) used to evaluate antibody responses was carried out as previously described [39]. Briefly, ELISA plates (Immunoplates, Nunc Maxisorb, Denmark) were coated with rabbit anti-IPNV designated as K95 [40] in coating buffer and were incubated overnight at 4 °C. After washing using PBS containing 20% Tween (PBST) thrice, plates were blocked using 5% dry milk at room temperature (RT) for 2 h. Thereafter, 0.5% formalin

inactivated IPNV antigen at a concentration of 1×10^5 $TCID_{50}$/mL was added to each well and incubated for 2 h at room temperature after washing thrice using PBST. Test plasma diluted in a twofold dilution starting from 1:10 to 1:320 was added to each well together with high and low positive controls as well as plasma and blank (1% dry milk PBST) controls. The plates were incubated overnight at 4 °C after adding diluted plasma samples. A mouse monoclonal anti-salmon antibody designated as 4C10 [41] targeting IgM heavy chain was diluted at 1:5000 and added to each well after washing three times using PBST. After incubation for 1 h, all plates were washed and goat-anti-mouse antibody conjugated with horse-radish peroxidase (DAKO; Glostrup, Denmark) was added to each well and incubated for 1 h at RT. After washing, substrate-containing OPD (O-phenylenediamine dihydrochloride, DAKO) constituted at manufacturer's recommendation was added to each well. Results were read using spectrophotometer (TECAN, Genios, Boston, USA) at 490 optic density wave length.

2.4. Organosomatic indices

Organosomatic indices (OSI) of the PI, MI and DI were calculated as the percentages of the weight of intestinal segments relative to the fish body weight; OSI = 100 * TW/BW, where TW is the tissue weight and BW is the fish body weight.

2.5. Histology

After fixation, PI, MI, and DI samples were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological techniques. The paraffin blocks were placed on a cooling block before cut by an automatic microtome (HM 355S, Thermo Scientific™, US) to produce sections of 3 μm thickness. The slides were then stained by hematoxylin and eosin, and examined blindly with a light microscope (Axio Scope.A1, Zeiss, Germany) equipped with a camera (AxioCam ICc 3, Zeiss) paying attention to typical inflammatory morphological changes observed in salmonid intestine fed soybean meal diets: that is, shortening and fusion of mucosal folds, cellular infiltration within the lamina propria and submucosa, enterocyte vacuolization and nucleus position disparity. Normally, little to no vacuolization is present in the enterocyte of the PI and MI whereas enterocytes of the DI show various degrees of supranuclear vacuolization that diminishes or disappears during the inflammation. For each histological characteristic evaluated, a value of normal, mild, moderate, marked or severe was assigned.

2.6. Quantitative real-time PCR

Real-time qPCR assays were performed following the MIQE guidelines [42]. Total RNA was extracted from PI and DI samples on a Biomek® 4000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA) using a Maxwell® HT simplyRNA Kit (Custom) (Premage, Madison, WI, USA) according to the manufacturer's protocol. RNA purity and concentration were measured using Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, VT, USA) and the integrity was verified by the 2100 Bioanalyzer with the 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, USA). The mean A_{260}/A_{280} ratio was 2.3 (S.D. = 0.01) and the average RIN (RNA integrity number) value was 8.4 (S.D. = 0.76). First-strand complementary DNA (cDNA) synthesis was performed using 1.0 μg total RNA from all samples using a Superscript™ IV VILO™ cDNA synthesis kit (catalog no., 11756050) (Invitrogen, Carlsbad, CA, USA). A negative control was set up by omitting RNA and the obtained cDNA was diluted 1:10 before use. The qPCR primers were obtained from the literature or designed using the Primer 3 (http://frodo.wi.mit.edu/primer3/). Primer efficiency was determined using 2-fold serial dilutions of randomly pooled cDNA. The qPCR assays were performed using the LightCycler 96 (Roche Applied Science, Basel, Switzerland) and a 10-μL reaction volume was used, which contained 2 μL of PCR-grade water, 2 μL diluted cDNA template, 5 μL LightCycler 480 SYBR Green I Master (Roche Applied Science) and 0.5 μL (10 μM) of each forward and reverse primer. Samples were run in duplicates in addition to a no-template control for each gene. A three-step qPCR programme was applied incorporating an enzyme activation step at 95 °C (5 min) and 45 cycles of 95 °C (10 s), 55–62 °C (10 s) and 72 °C (15 s). The plate pipetting was done using the Biomek® 4000 automation workstation. Quantification cycle (Cq) values were determined using the second derivative method. The specificity of qPCR amplification was confirmed by evaluating the melting curve of qPCR products and the band pattern on the agarose gel after electrophoresis. Beta-actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase 2 (*rnapo2*) and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) were evaluated for use as reference genes according to their stability across and within the treatments [43]. The expression of target genes in the PI was normalized to the geometric mean of *actb*, *rnapo2* and *hprt1*. For DI samples, the geometric mean of *gapdh* and *rnapo2* was used for the normalization. The mean normalized expression of the target genes was calculated from raw Cq values [44]. The genes profiled and the primers used for the qPCR assays are given in Table S1.

2.7. Statistics

Unless specified, statistical analyses were performed in JMP Pro 13.0.0 (SAS Institute, United States). For continuous variables, data were fitted to linear mixed model treating diet as fixed effect and tank as random effect. The homoscedasticity and normality of residuals was assessed visually by checking the “residual by predicted” plot and histogram, respectively. When necessary, data were subjected to box-cox power transformation to meet the model assumptions and refitted again. For ordinal variables, data were initially fitted to cumulative link mixed model implemented by the R package *ordinal* [45], treating diet as fixed effect and tank as random effect. As the full model resulted in high Hessian number and the random effect was not significant, the tank effect was dropped from the full model. The proportional odds assumption was checked by the nominal test. Spearman's rank correlation analysis was carried out to discover associations between variables of interests. Multiple comparisons were adjusted by the Bonferroni correction or adaptive two-stage Benjamini-Hochberg procedure where applicable. Data were presented as mean ± SEM. Statistically significant results were indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3. Result

To aid readers in interpreting data reported here, results on general fish performance and nutrients utilization, which have been published elsewhere [34], are summarized below.

In brief, no differences between the diet groups were recorded for feed intake, feed conversion ratio, body weight gain or protein productive value, despite the test group showing a lower protein digestibility. Higher condition factor, hepatosomatic and viscerosomatic indices were observed in fish fed the IM based diet. Regarding gut function, no diet effect on chyme trypsin activity or bile salt level was noted, but the brush border enzyme, leucine aminopeptidase, showed lower activity in the IM diet group.

3.1. Antibody responses

No significant difference was observed between the two groups for the plasma antibody level against IPNV ($p > 0.05$) (Fig. 1).

3.2. Somatic indices of intestinal sections

No significant diet effect was observed for PI-somatic index (PISI).

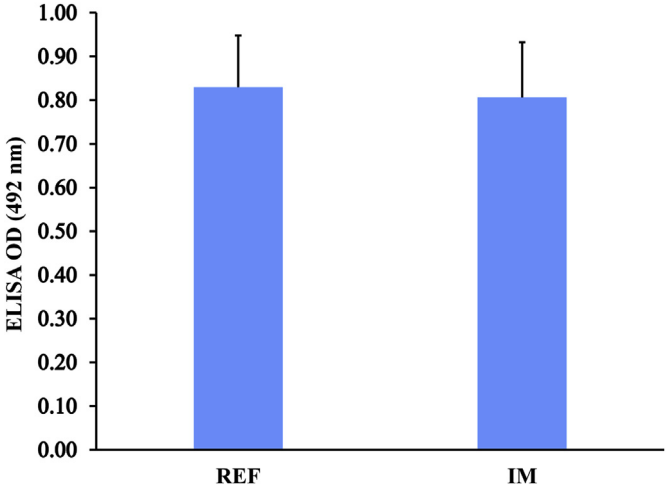

Fig. 1. Plasma antibody level against IPNV of salmon presmolts fed the experimental diets. REF, reference diet; IM, insect meal test diet. The results were obtained from plasma diluted 1:40.

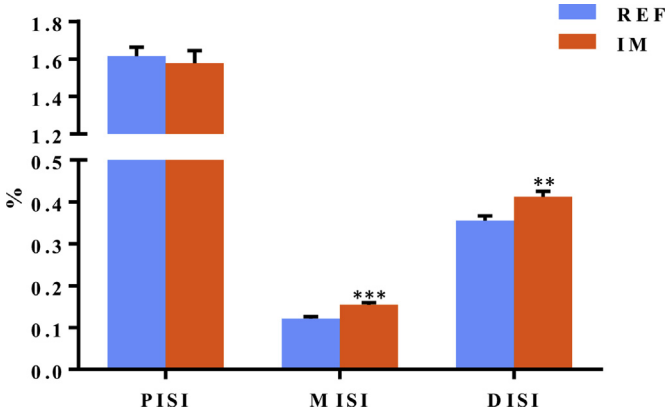

Fig. 2. Intestine-somatic indices of salmon presmolts fed the experimental diets. PISI, proximal intestine-somatic index; MISI, mid intestine-somatic index; DISI, distal intestine-somatic index. REF, reference diet; IM, insect meal test diet. Asterisks denote statistically significant differences between the diets (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

However, both MI-somatic index (MISI) and DI-somatic index (DISI) were significantly higher in fish fed the IM diet (Fig. 2) compared to those fed the reference diet.

3.3. Histological appearance

In the PI, increased vacuolization or hyper-vacuolization of the enterocytes, suggestive of lipid accumulation (steatosis), was observed in fish from both diet groups, but was significantly less prevalent in fish fed the IM diet (Fig. 3). The MI and DI showed normal and healthy histological structure for all sampled fish.

3.4. Gene expression

The results are presented as fold change of gene expression level in fish fed the IM diet relative to those fed the REF diet (Fig. 4). Correlations between fold changes of functionally-related genes are shown in Table 2.

In the PI, IM inclusion had minor effects on the expression profile of immune genes. The exception was the increase in expression of *cd3*$_{\gamma\delta}$ and *foxp3*. Similarly, no effect of diet was observed on the expression of tight junction genes *cldn15* or *cldn25b*. In support of the histological observation, there was a 2-fold decrease in the expression of *plin2*, a

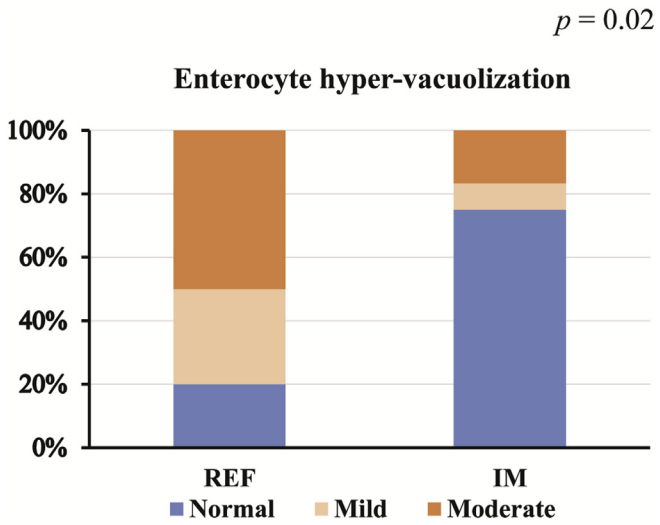

Fig. 3. Contingency chart showing proportions of sampled individuals scored normal, mild, or moderate (none scored above moderate) regarding hyper-vacuolization of enterocytes in the PI. REF, reference diet; IM, insect meal test diet. The *p* value of the diet effect was given. For the illustration of enterocyte hyper-vacuolization at various degrees, one can refer to Fig. S1.

surface marker of lipid droplets. Genes involved in cholesterol uptake (*npc1l1*) and de novo synthesis (*srebp2* and *cyp51*) showed increased expression, and so were those responsible for the absorption (*cd36*) and intracellular transportation (*fabp2b*) of fatty acids. Most of the detoxification response relevant genes were up-regulated, which included *cyp1a1* (a marker for xenobiotic metabolism), *mta* (a marker for heavy metal detoxification), *hsp70* (a marker for stress response), *sod1* (an indicator of oxidative stress) and *cat* (an indicator of oxidative stress). However, no correlations were found between *cyp1a1* and other genes profiled under the same category.

In the DI, less differences in the gene expression were observed, most of which showed similar responses to those observed in the PI. These genes included *cd3*$_{\gamma\delta}$, *foxp3*, *pcna*, *cyp1a1* and *cat*. Notably, the increased expression of *cd3*$_{\gamma\delta}$ showed a strong positive correlation with that of *foxp3*. *Cyp1a1* also showed an increase in the mRNA level of more than 2 folds in fish fed the IM diet. In contrast to what we found in the PI, the expression of *plin2* was increased, coinciding with a marked induction of *apoa4* expression.

4. Discussion

In summary, the main findings of the present study showed that dietary inclusion of IM as high as 60% did not cause appreciable negative effects on the gut health of Atlantic salmon. Its inclusion, however, reduced enterocyte hyper-vacuolization in the pyloric caeca, and seemed to stimulate regulatory T cell activity as well as xenobiotic metabolism both in the proximal and distal intestine.

The inclusion of certain legume-based alternative feed ingredients in salmon feed is known to cause proliferative or inflammatory conditions in the intestinal mucosa, resulting in compromised fish health and welfare [4,27]. In the present study, no signs of local inflammatory response to the IM inclusion was noted. Insect meal inclusion seemed however to stimulate regulatory T cell activity both in the proximal and distal intestine, as indicated by the increased expression of both *cd3*$_{\gamma\delta}$ and *foxp3* in these gut segments. It has been shown that the expression pattern of some cytokines that enhance T-cell responses are prone to induce oral tolerance to vaccination while others are not [46]. As a transcription factor for the differentiation of naïve CD4 T-cells into T-regulatory genes (T-regs), *foxp3* is the main marker of oral tolerance induced by prolonged exposure to the same antigenic proteins through

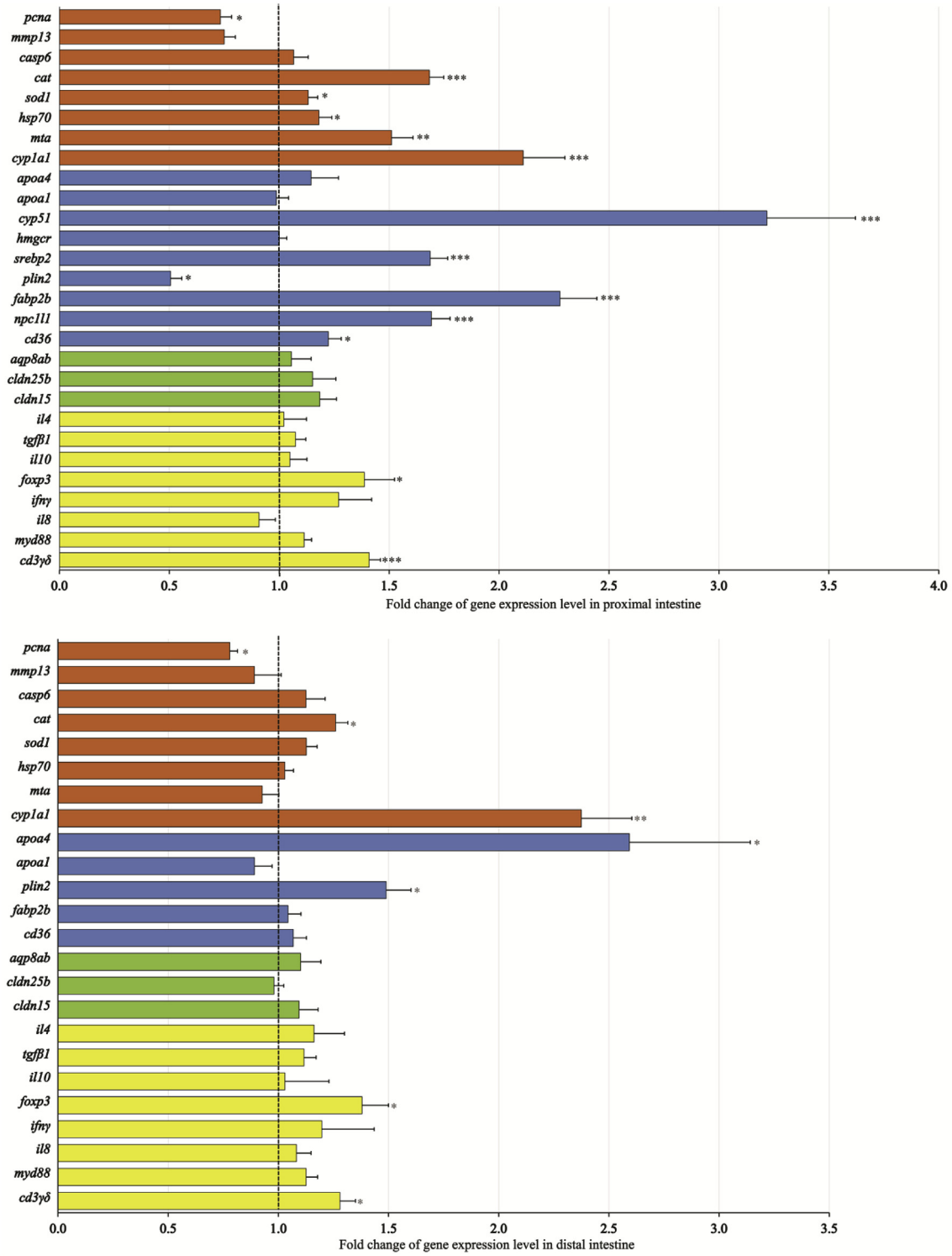

Fig. 4. The fold change of gene expression level in proximal and distal intestine of fish fed the IM diet relative to those fed the REF diet. The fold change was calculated by dividing the gene expression level of individual fish fed the IM diet by the mean expression level of fish fed the REF diet. Hence, the error bar indicates the variation in gene expression level in fish fed the IM diet. Values > 1 indicate an increased gene expression level in the IM fed fish, and vice versa. Genes relevant to immune response, barrier function, lipid metabolism and xenobiotic metabolism are shaded in yellow, green, blue and orange, respectively. For the explanation of gene abbreviations, see Table S1. *P* values of test statistics were adjusted by the Benjamini-Hochberg procedure for the proximal and distal intestine, respectively. Asterisks denote statistically significant differences between the diets (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the feed. Its overexpression has been linked to significant loss of systemic immune responses to vaccination [47,48]. As pointed out by other scientists, oral tolerance through feed can be initiated by tolerogenic protein molecules [49–51]. Oral tolerance following vaccination has been reported in different fish species and it can reduce immune protection [52–56]. Hence, there is a need for follow-up studies using oral vaccines to determine whether the larvae meal from black soldier fly produces tolerogenic proteins that evoke increased expression levels

Table 2
Correlations between fold changes of functionally related genes*.

Variables of interest	Spearman ρ	*p* value	Adjusted *p* value
PI			
cd3$_{\gamma\delta}$ & foxp3	0.63	0.0280	0.0583
cd36 & fabp2b	0.15	0.6482	0.5834
cyp1a1 & mta	0.11	0.7452	0.5868
cyp1a1 & hsp70	0.28	0.3715	0.4393
cyp1a1 & sod1	−0.61	0.0370	0.0583
cyp1a1 & cat	−0.26	0.4184	0.4393
sod1 & cat	0.87	0.0002	0.0013
srebp2 & cyp51	0.80	0.0019	0.0060
DI			
cd3$_{\gamma\delta}$ & foxp3	0.95	0.0001	0.0003
cyp1a1 & cat	0.12	0.7129	1.0000
apoa4 & plin2	0.34	0.2861	0.8583

*p values of test statistics for the PI were adjusted by the Benjamini-Hochberg procedure whereas those for the DI were adjusted by the Bonferroni correction. PI, proximal intestine; DI, distal intestine.

of *foxp3*, which could culminate in tolerance. On the other hand, previous studies have shown divergent and size-dependent effects of chitin on immune functions in mice cell models [57]. Small chitin molecules (< 40 μm), in particular, have been observed to induce production of anti-inflammatory cytokine *il10* [58]. In most cases, *il10* upregulation corroborates with increased expression of *tgf-β*, which is a regulatory cytokine for *foxp3* in T-regs. Whether the increased expression of *cd3$_{\gamma\delta}$* and *foxp3* may be associated with exposure to chitin, given that chitin has tolerogenic properties, deserves further investigation.

Dietary composition has been shown to influence the outcome of antibody responses in vaccinated fish. For example, Burrells et al. [59] showed elevated antibody responses against *Aeromonas salmonicida* in a diet containing novel protein supplements added to a standard commercial diet (EWOS VEXTRA Alpha®). On the contrary, Erdal et al. [60] observed decreased antibody levels against *Yersinia ruckeri* in fish fed higher levels of dietary ω-3 fatty acids. In the present study, there was no significant difference observed between the groups fed the REF and IM diets in antibody response against IPNV. These findings suggest that the two feeds have a similar impact on the inactivated whole vaccine against IPNV and that replacing the REF diet with the IM diet would not alter the protective ability of the vaccines. Given the increasing demand for oral vaccines in salmon aquaculture, future studies should investigate the effect of the BSF diet on enhancing mucosal immunity using oral vaccines.

The proximal intestine of IM-fed fish displayed increased expression of genes involved in uptake of fatty acids (*cd36*, *fabp2*) and cholesterol uptake and de novo synthesis (*npc1l1*, *srebp2*, *cyp51*). Associations between differential expression of fatty acid uptake genes and reductions of enterocyte hyper-vacuolization have been reported previously [61]. Our observations in the current work could reflect a generally increased uptake, turnover and transport of fatty acids in IM-fed fish. Several previous studies on salmon have also demonstrated the close relationship between dietary sterol levels and expression of genes related to cholesterol uptake and synthesis [61–64]. In the present study, the increased expression of genes related to cholesterol metabolism was likely caused by the different sterol composition of the two feeds, in which the IM diet contained about 50% less cholesterol but much higher phytosterol levels than the REF diet (unpublished data).

The use of plant ingredients in Atlantic salmon diets has been linked to increased hyper-vacuolization of enterocytes in the proximal intestine [61]. This hyper-vacuolization is apparently reflecting an abnormal lipid droplet accumulation within the enterocytes, i.e. steatosis [65–67]. The mechanism underlying the intracellular lipid accumulation is currently unknown, but has previously been proposed to involve a disrupted assembly of lipoproteins, possibly caused by lack of major building blocks, such as cholesterol, phosphatidylcholine and/or apolipoproteins [61]. In severe cases, the hyper-vacuolization may be accompanied by large accumulations of lipidic material in the intestinal chyme and is referred to as lipid malabsorption, eventually resulting in steatorrhea and the so-called “floating feces” on the surface of sea cages [28,29]. In the current study, the replacement of fishmeal and plant-based protein sources with IM resulted in less lipid accumulation within proximal intestinal enterocytes, observed as less prevalent enterocyte hyper-vacuolization. This was reflected in the marked suppression of *plin2* (also known as adipophilin) mRNA levels, a surface marker of lipid droplets [68], in the proximal intestine of fish fed the IM based diet. The reason for the reduction in enterocyte hyper-vacuolization in IM-fed fish is not clear but could be related to lower levels of plant ingredients (i.e. SPC and rapeseed oil) and/or actions of certain bioactive components present in the IM. It should be noted that there were no macroscopic appearances of lipid malabsorption in any fish at the time of sampling, and no apparent indications of reduced fish health as a result of the hyper-vacuolization.

To assess the exposure to toxic substances in fish, some useful biomarkers have been suggested which include, but are not limited to, biotransformation enzymes (Cyp1a1), anti-oxidant enzymes (Sod, Cat), stress proteins (Hsp70) and metallothioneins (Mta; a specific marker for exposure to metals) [69]. Of note, the phase I biotransformation enzyme, Cyp1a, is among the most sensitive biomarkers and has been validated in numerous studies in fish including Atlantic salmon [70–72]. In the present study, a more than 2-fold increase in the expression level of *cyp1a1* was found in the distal intestine of IM fed fish, while other marker genes showed no expression differences. Despite a lack of significant correlations in the expression levels between *cyp1a1* and other marker genes, their increased mRNA levels in the proximal intestine of fish fed the IM diet is indicative of elevated detoxification response to the diet. The responses may partially associate with the higher concentration of certain heavy metals in the IM diet such as nickel, cadmium, and lead [34], the latter two of which were found to be selectively enriched in the BSF larvae from the growing substrate [73–75]. However, the consequences on gut health of increased exposure to heavy metals seemed mild, as no significant diet effects were observed for toxicity indicators such as *casp6* (cell apoptosis) and *mmp13* (tissue regeneration), a finding in agreement with the absence of abnormalities on histological appearance and organ indices of the gut tissue.

In conclusion, our results showed no indications that the insect meal-based diet negatively affected the gut health of Atlantic salmon. On the contrary, the diet tended to improve gut function by reducing excessive lipid deposition within the enterocytes of the pyloric caeca. Stimulation of xenobiotic metabolism in the proximal intestine could possibly be a response to increased exposure to heavy metals selectively enriched by BSF larvae, such as cadmium and lead. Upregulation of Foxp3, a molecular marker for immune tolerance, calls for further investigations using oral vaccines to determine whether BSF has tolerogenic properties. The similarity in plasma IgM levels against IPNV indicated that BSF can safely replace fishmeal without inducing adverse effects that would reduce antibody responses for parenteral vaccines.

Conflicts of interest

The authors declare no competing financial interest.

Funding

This work is part of the “AquaFly” project (grant number, 238997), funded by the Research Council of Norway. Other costs related to this study were covered by the NMBU. The funding agencies had no role in study design, data collection, and interpretation, decision to publish or preparation of the manuscript.

Acknowledgements

Y. L. is pursuing his Ph.D. degree at NMBU with a scholarship granted by the China Scholarship Council, a non-profit organization sponsoring Chinese citizens to study abroad. The authors gratefully acknowledge animal technicians at the EWOS research station at Dirdal, Norway, for their committed animal care and Ellen K. Hage for organizing the sampling and the skillful lab work and leadership.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.057.

References

[1] T. Ytrestøyl, T.S. Aas, T. Åsgård, Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway, Aquaculture 448 (2015) 365–374.
[2] Å. Krogdahl, A. Bakke-McKellep, K. Roed, G. Baeverfjord, Feeding Atlantic salmon *Salmo salar* L. soybean products: effects on disease resistance (furunculosis), and lysozyme and IgM levels in the intestinal mucosa, Aquacult. Nutr. 6 (2) (2000) 77–84.
[3] D. Knudsen, F. Jutfelt, H. Sundh, K. Sundell, W. Koppe, H. Frøkiær, Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (*Salmo salar* L.), Br. J. Nutr. 100 (01) (2008) 120–129.
[4] M.H. Penn, E.Å. Bendiksen, P. Campbell, Å. Krogdahl, High level of dietary pea protein concentrate induces enteropathy in Atlantic salmon (*Salmo salar* L.), Aquaculture 310 (3) (2011) 267–273.
[5] A.M. Bakke-McKellep, C.M. Press, G. Baeverfjord, Å. Krogdahl, T. Landsverk, Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis, J. Fish. Dis. 23 (2) (2000) 115–127.
[6] C.J. Shepherd, O. Monroig, D.R. Tocher, Future availability of raw materials for salmon feeds and supply chain implications: the case of Scottish farmed salmon, Aquaculture 467 (2017) 49–62.
[7] M. Øverland, A. Skrede, Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture, J. Sci. Food Agric. 97 (3) (2016) 733–742.
[8] M.-J. Sánchez-Muros, F.G. Barroso, F. Manzano-Agugliaro, Insect meal as renewable source of food for animal feeding: a review, J. Clean. Prod. 65 (2014) 16–27.
[9] A. van Huis, Potential of insects as food and feed in assuring food security, Annu. Rev. Entomol. 58 (2013) 563–583.
[10] A. Van Huis, J. Van Itterbeeck, H. Klunder, E. Mertens, A. Halloran, G. Muir, P. Vantomme, Edible Insects: Future Prospects for Food and Feed Security, Food and Agriculture Organization of the United Nations, 2013.
[11] G. Burtle, G.L. Newton, D.C. Sheppard, T. Campus, Mass Production of Black Soldier Fly Pre pupae for Aquaculture Diets. Preprint, accessed on 2018 Nov 15. Available from: http://www.caes.uga.edu/content/dam/caes-website/extension-outreach/commodities/aquaculture/docs/soldier-fly-magazine-article.pdf.
[12] G. Newton, C. Booram, R. Barker, O. Hale, Dried Hermetia illucens larvae meal as a supplement for swine, J. Anim. Sci. 44 (3) (1977) 395–400.
[13] S. St-Hilaire, C. Sheppard, J.K. Tomberlin, S. Irving, L. Newton, M.A. McGuire, E.E. Mosley, R.W. Hardy, W. Sealey, Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*, J. World Aquacult. Soc. 38 (1) (2007) 59–67.
[14] F.G. Barroso, C. de Haro, M.-J. Sánchez-Muros, E. Venegas, A. Martínez-Sánchez, C. Pérez-Bañón, The potential of various insect species for use as food for fish, Aquaculture 422–423 (2014) 193–201.
[15] W.M. Sealey, T.G. Gaylord, F.T. Barrows, J.K. Tomberlin, M.A. McGuire, C. Ross, S. St-Hilaire, Sensory analysis of rainbow trout, *Oncorhynchus mykiss*, fed enriched black soldier fly prepupae, *Hermetia illucens*, J. World Aquacult. Soc. 42 (1) (2011) 34–45.
[16] M. Renna, A. Schiavone, F. Gai, S. Dabbou, C. Lussiana, V. Malfatto, M. Prearo, M.T. Capucchio, I. Biasato, E. Biasibetti, M. De Marco, A. Brugiapaglia, I. Zoccarato, L. Gasco, Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss* Walbaum) diets, J. Anim. Sci. Biotechnol. 8 (57) (2017).
[17] R. Magalhaes, A. Sanchez-Lopez, R.S. Leal, S. Martinez-Llorens, A. Oliva-Telesa, H. Peres, Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*), Aquaculture 476 (2017) 79–85.
[18] S.L. Li, H. Ji, B.X. Zhang, J.J. Tian, J.S. Zhou, H.B. Yu, Influence of black soldier fly (*Hermetia illucens*) larvae oil on growth performance, body composition, tissue fatty acid composition and lipid deposition in juvenile Jian carp (*Cyprinus carpio* var. Jian), Aquaculture 465 (2016) 43–52.
[19] S.L. Li, H. Ji, B.X. Zhang, J.S. Zhou, H.B. Yu, Defatted black soldier fly (*Hermetia illucens*) larvae meal in diets for juvenile Jian carp (*Cyprinus carpio* var. Jian): growth performance, antioxidant enzyme activities, digestive enzyme activities, intestine and hepatopancreas histological structure, Aquaculture 477 (2017) 62–70.
[20] S. Kroeckel, A.G.E. Harjes, I. Roth, H. Katz, S. Wuertz, A. Susenbeth, C. Schulz, When a turbot catches a fly: evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute — growth performance and chitin degradation in juvenile turbot (*Psetta maxima*), Aquaculture 364–365 (2012) 345–352.
[21] J.R. Hu, G.X. Wang, Y.H. Huang, Y.P. Sun, F. He, H.X. Zhao, N.N. Li, Effects of substitution of fish meal with black soldier fly (*Hermetia illucens*) larvae meal, in yellow catfish (*Pelteobagrus fulvidraco*) diets, Isr. J. Aquacult. Bamidgeh 69 (2017).
[22] E. Devic, W. Leschen, F. Murray, D.C. Little, Growth performance, feed utilization and body composition of advanced nursing Nile tilapia (*Oreochromis niloticus*) fed diets containing Black Soldier Fly (*Hermetia illucens*) larvae meal, Aquacult. Nutr. 24 (1) (2017) 416–423.
[23] M. Borgogno, C. Dinnella, V. Iaconisi, R. Fusi, C. Scarpaleggia, A. Schiavone, E. Monteleone, L. Gasco, G. Parisi, Inclusion of *Hermetia illucens* larvae meal on rainbow trout (*Oncorhynchus mykiss*) feed: effect on sensory profile according to static and dynamic evaluations, J. Sci. Food Agric. 97 (10) (2017) 3402–3411.
[24] K. Bondari, D. Sheppard, Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (Rafinesque), and blue tilapia, *Oreochromis aureus* (Steindachner), Aquacult. Fish. Manag. 18 (3) (1987) 209–220.
[25] E.R. Lock, T. Arsiwalla, R. Waagbo, Insect larvae meal as an alternative source of nutrients in the diet of Atlantic salmon (*Salmo salar*) postsmolt, Aquacult. Nutr. 22 (6) (2016) 1202–1213.
[26] O.B. Dale, B. Torud, A. Kvellestad, H.S. Koppang, E.O. Koppang, From chronic feed-induced intestinal inflammation to adenocarcinoma with metastases in salmonid fish, Cancer Res. 69 (10) (2009) 4355–4362.
[27] G. Baeverfjord, Å. Krogdahl, Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L, distal intestine: a comparison with the intestines of fasted fish, J. Fish. Dis. 19 (5) (1996) 375–387.
[28] R. Hanche-Olsen, L. Brunvold, M. Hillestad, H. Lysne, A.D. Løland, M. Penn, Sluttrapport: Nedsatt Tarmhelse Og Forekomst Av Flytefeces Hos Laks (In Norwegian), Helgeland Havbruksstasjon, 2013, https://www.fhf.no/prosjektdetaljer/?projectNumber=900722 , Accessed date: 21 May 2018.
[29] M.H. Penn, Lipid Malabsorption in Atlantic Salmon-the Reoccurring Problem of Floating Feces, Fiskehelse, Tekna Fiskehelseforeningen, Oslo, 2011, pp. 6–11.
[30] C.A. Da Silva, D. Hartl, W. Liu, C.G. Lee, J.A. Elias, TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation, J. Immunol. 181 (6) (2008) 4279–4286.
[31] M. Henry, L. Gasco, G. Piccolo, E. Fountoulaki, Review on the use of insects in the diet of farmed fish: past and future, Anim. Feed Sci. Technol. 203 (2015) 1–22.
[32] E. Ringo, Z. Zhou, R.E. Olsen, S.K. Song, Use of chitin and krill in aquaculture - the effect on gut microbiota and the immune system: a review, Aquacult. Nutr. 18 (2) (2012) 117–131.
[33] M.W. Ferreira, F.G. de Araujo, D.V. Costa, P.V. Rosa, H.C. Figueiredo, L.D. Murgas, Influence of dietary oil sources on muscle composition and plasma lipoprotein concentrations in Nile Tilapia, *Oreochromis niloticus*, J. World Aquacult. Soc. 42 (1) (2011) 24–33.
[34] I. Belghit, N.S. Liland, R. Waagbo, I. Biancarosa, N. Pelusio, Y.X. Li, Å. Krogdahl, E.J. Lock, Potential of insect-based diets for Atlantic salmon (*Salmo salar*), Aquaculture 491 (2018) 72–81.
[35] H.M. Munang'andu, B.N. Fredriksen, S. Mutoloki, R.A. Dalmo, Ø. Evensen, The kinetics of CD4+ and CD8+ T-cell gene expression correlate with protection in Atlantic salmon (*Salmo salar* L) vaccinated against infectious pancreatic necrosis, Vaccine 31 (15) (2013) 1956–1963.
[36] Q. Qin, T. Wu, T. Jia, A. Hegde, R. Zhang, Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus, J. Virol. Methods 131 (1) (2006) 58–64.
[37] H.M. Munang'andu, B.N. Fredriksen, S. Mutoloki, R.A. Dalmo, Ø. Evensen, Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L), Vet. Res. 44 (1) (2013) 7.
[38] H.M. Munang'andu, N. Santi, B.N. Fredriksen, K.-E. Løkling, Ø. Evensen, A systematic approach towards optimizing a cohabitation challenge model for infectious pancreatic necrosis virus in Atlantic salmon (*Salmo salar* L.), PLoS One 11 (2) (2016) e0148467.
[39] H.M. Munang'andu, B.N. Fredriksen, S. Mutoloki, B. Brudeseth, T.-Y. Kuo, I.S. Marjara, R.A. Dalmo, Ø. Evensen, Comparison of vaccine efficacy for different antigen delivery systems for infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L.) in a cohabitation challenge model, Vaccine 30 (27) (2012) 4007–4016.
[40] Ø. Evensen, E. Rimstad, Immunohistochemical identification of infectious pancreatic necrosis virus in paraffin-embedded tissues of Atlantic salmon (*Salmo salar*), J. Vet. Diagn. Invest. 2 (4) (1990) 288–293.
[41] A. Thuvander, C. Fossum, N. Lorenzen, Monoclonal antibodies to salmonid immunoglobulin: characterization and applicability in immunoassays, Dev. Comp. Immunol. 14 (4) (1990) 415–423.
[42] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (4) (2009) 611–622.
[43] T.M. Kortner, E.C. Valen, H. Kortner, I.S. Marjara, Å. Krogdahl, A.M. Bakke, Candidate reference genes for quantitative real-time PCR (qPCR) assays during development of a diet-related enteropathy in Atlantic salmon (*Salmo salar* L.) and the potential pitfalls of uncritical use of normalization software tools, Aquaculture 318 (3–4) (2011) 355–363.
[44] P.Y. Muller, H. Janovjak, A.R. Miserez, Z. Dobbie, Processing of gene expression data generated by quantitative real-time RT-PCR, Biotechniques 32 (6) (2002) 1372–1374 1376, 1378-9.
[45] R.H.B. Christensen, ordinal-Regression Models for Ordinal Data. R package version, (2018), pp. 8–25 http://www.cran.r-project.org/package=ordinal/.

[46] K.S. Barone, D.D. Tolarova, I. Ormsby, T. Doetschman, J.G. Michael, Induction of oral tolerance in TGF-β1 null mice, J. Immunol. 161 (1) (1998) 154–160.
[47] F. Ramsdell, Foxp3 and natural regulatory T cells: key to a cell lineage? Immunity 19 (2) (2003) 165–168.
[48] M.G. Roncarolo, S. Gregori, Is FOXP3 a bona fide marker for human regulatory T cells? Eur. J. Immunol. 38 (4) (2008) 925–927.
[49] A.M. Mowat, A.G. Lamont, M.G. Bruce, A genetically determined lack of oral tolerance to ovalbumin is due to failure of the immune system to respond to intestinally derived tolerogen, Eur. J. Immunol. 17 (11) (1987) 1673–1676.
[50] O. Pabst, A. Mowat, Oral tolerance to food protein, Mucosal Immunol. 5 (3) (2012) 232–239.
[51] S. Strobel, A.M. Mowat, Immune responses to dietary antigens: oral tolerance, Immunol. today 19 (4) (1998) 173–181.
[52] J. Rombout, A. Berg, C. Berg, P. Witte, E. Egberts, Immunological importance of the second gut segment of carp. III. Systemic and/or mucosal immune responses after immunization with soluble or particulate antigen, J. Fish. Biol. 35 (2) (1989) 179–186.
[53] P. Joosten, M. Engelsma, M. Van der Zee, J. Rombout, Induction of oral tolerance in carp (*Cyprinus carpio* L.) after feeding protein antigens, Vet. Immunol. Immunopathol. 60 (1–2) (1997) 187–196.
[54] G. Davidson, A. Ellis, C. Secombes, A preliminary investigation into the phenomenon of oral tolerance in rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792), Fish Shellfish Immunol. 4 (2) (1994) 141–151.
[55] H.M. Munang'andu, S. Mutoloki, Ø. Evensen, An overview of challenges limiting the design of protective mucosal vaccines for finfish, Front. Immunol. 6 (2015) 542.
[56] S. Maurice, A. Nussinovitch, N. Jaffe, O. Shoseyov, A. Gertler, Oral immunization of *Carassius auratus* with modified recombinant A-layer proteins entrapped in alginate beads, Vaccine 23 (4) (2004) 450–459.
[57] C.G. Lee, C.A. Da Silva, J.-Y. Lee, D. Hartl, J.A. Elias, Chitin regulation of immune responses: an old molecule with new roles, Curr. Opin. Immunol. 20 (6) (2008) 684–689.
[58] C.A. Da Silva, C. Chalouni, A. Williams, D. Hartl, C.G. Lee, J.A. Elias, Chitin is a size-dependent regulator of macrophage TNF and IL-10 production, J. Immunol. 182 (6) (2009) 3573–3582.
[59] C. Burrells, P. Williams, P. Southgate, S. Wadsworth, Dietary nucleotides: a novel supplement in fish feeds: 2. Effects on vaccination, salt water transfer, growth rates and physiology of Atlantic salmon (*Salmo salar* L.), Aquaculture 199 (1–2) (2001) 171–184.
[60] J.I. Erdal, Ø. Evensen, O.K. Kaurstad, A. Lillehaug, R. Solbakken, K. Thorud, Relationship between diet and immune response in Atlantic salmon (*Salmo salar* L.) after feeding various levels of ascorbic acid and omega-3 fatty acids, Aquaculture 98 (4) (1991) 363–379.
[61] M. Gu, T.M. Kortner, M. Penn, A.K. Hansen, Å. Krogdahl, Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.), Br. J. Nutr. 111 (03) (2014) 432–444.
[62] N.S. Liland, M. Espe, G. Rosenlund, R. Waagbø, J.I. Hjelle, Ø. Lie, R. Fontanillas, B.E. Torstensen, High levels of dietary phytosterols affect lipid metabolism and increase liver and plasma TAG in Atlantic salmon (*Salmo salar* L.), Br. J. Nutr. 110 (11) (2013) 1958–1967.
[63] T.M. Kortner, I. Björkhem, A. Krasnov, G. Timmerhaus, Å. Krogdahl, Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.), Br. J. Nutr. 111 (12) (2014) 2089–2103.
[64] T.M. Kortner, J. Gu, Å. Krogdahl, A.M. Bakke, Transcriptional regulation of cholesterol and bile acid metabolism after dietary soyabean meal treatment in Atlantic salmon (*Salmo salar* L.), Br. J. Nutr. 109 (04) (2013) 593–604.
[65] R. Olsen, R. Myklebust, T. Kaino, E. Ringø, Lipid digestibility and ultrastructural changes in the enterocytes of Arctic char (*Salvelinus alpinus* L.) fed linseed oil and soybean lecithin, Fish Physiol. Biochem. 21 (1) (1999) 35–44.
[66] R. Olsen, R. Myklebust, E. Ringø, T. Mayhew, The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Arctic char (*Salvelinus alpinus* L.): a quantitative ultrastructural study, Fish Physiol. Biochem. 22 (3) (2000) 207–216.
[67] R.E. Olsen, B.T. Dragnes, R. Myklebust, E. Ringø, Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, *Oncorhynchus mykiss* Walbaum, Fish Physiol. Biochem. 29 (3) (2003) 181–192.
[68] H. Heid, R. Moll, I. Schwetlick, H.-R. Rackwitz, T.W. Keenan, Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases, Cell Tissue Res. 294 (2) (1998) 309–321.
[69] R. van der Oost, J. Beyer, N.P. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ. Toxicol. Pharmacol. 13 (2) (2003) 57–149.
[70] M. Sanden, P.A. Olsvik, Intestinal cellular localization of PCNA protein and CYP1A mRNA in Atlantic salmon *Salmo salar* L. exposed to a model toxicant, BMC Physiol. 9 (2009) 3.
[71] A.S. Mortensen, A. Arukwe, Modulation of xenobiotic biotransformation system and hormonal responses in Atlantic salmon (*Salmo salar*) after exposure to tributyltin (TBT), Comp. Biochem. Physiol. C Toxicol. Pharmacol. 145 (3) (2007) 431–441.
[72] A.S. Mortensen, A. Arukwe, Effects of 17 alpha-ethynylestradiol on hormonal responses and xenobiotic biotransformation system of Atlantic salmon (*Salmo salar*), Aquat. Toxicol. 85 (2) (2007) 113–123.
[73] M. Tschirner, A. Simon, Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed, J. Insects Food Feed 1 (4) (2015) 249–259.
[74] B. Purschke, R. Scheibelberger, S. Axmann, A. Adler, H. Jager, Impact of substrate contamination with mycotoxins, heavy metals and pesticides on the growth performance and composition of black soldier fly larvae (*Hermetia illucens*) for use in the feed and food value chain, Food Addit. Contam. A: Chem. Anal. Contr. Exposure Risk Assess. 34 (8) (2017) 1410–1420.
[75] S. Diener, C. Zurbrügg, K. Tockner, Bioaccumulation of heavy metals in the black soldier fly, *Hermetia illucens* and effects on its life cycle, J. Insects Food Feed 1 (4) (2015) 261–270.