Fish and Shellfish Immunology 91 (2019) 223–232

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Effect of dietary replacement of fish meal with insect meal on *in vitro* bacterial and viral induced gene response in Atlantic salmon (*Salmo salar*) head kidney leukocytes

Oda Kvalsvik Stenberg[a], Elisabeth Holen[a], Luisa Piemontese[a,b], Nina S. Liland[a], Erik-Jan Lock[a], Marit Espe[a], Ikram Belghit[a,*]

[a] Institute of Marine Research, P.O. Box 1870 Nordnes, 5817, Bergen, Norway
[b] Department of Agriculture, Food, Environment and Forestry, University of Florence, Via delle Cascine 5, 50144, Firenze, Italy

ARTICLE INFO

Keywords:
Head kidney leukocytes
Insect meal
Stress response
LPS
Poly I
C
Atlantic salmon

ABSTRACT

With the fast growth of today's aquaculture industry, the demand for aquafeeds is expanding dramatically. Insects, which are part of the natural diet of salmonids, could represent a sustainable ingredient for aquaculture feed. The aim of the current study was to test how a partial or total replacement of dietary fishmeal with insect meal affect gene responses involved in inflammation, the eicosanoid pathway and stress response in Atlantic salmon (*Salmo salar* L.) in isolated head kidney leukocytes after exposure to bacterial or viral mimic.

Insect meal (IM) was produced from black soldier fly (BSF, *Hermetia illucens*) larvae. Seawater Atlantic salmon were fed three different diets for 8 weeks; a control diet (IM_0, protein from fishmeal and plant based ingredients (25:75) and lipid from fish oil and vegetable oil (33:66); and two insect-meal containing diets, IM_{66} and IM_{100}, where 66 and 100% of the fishmeal protein was replaced with IM, respectively. Leukocytes were isolated from the head kidney of fish (n = 6) from each of the three dietary groups. Isolated leukocytes were seeded into culture wells and added either a bacterial mimic (lipopolysaccharide, LPS) or a viral mimic (polyinosinic acid: polycytidylic acid, poly I: C) to induce an inflammatory response. Controls (Ctl) without LPS and poly I: C were included.

The transcription of interleukins *IL-1β*, *IL-8*, *IL-10* and *TNF-α* were elevated in LPS treated leukocytes isolated from salmon fed the three dietary groups (IM_0, IM_{66} and IM_{100}). The inflammatory-related gene expression in head kidney cells were, however, not affected by the pre-fed substitution of fish meal with IM in the diet of salmon. Gene transcriptions of *PTGDS* and *PTGES* were neither affected by LPS, poly I: C or the experimental diets fed prior to cell isolation, while salmon fed with IM showed a lower expression of *LOX5*. The gene expression of *TLR22* and *C/EBP-β* were down-regulated by the LPS treatment in the cells isolated from salmon fed insect-based diets (IM_{66} and IM_{100}) compared to fish fed the IM_0. Similarly, the leukocytes challenged with LPS and isolated from fish fed with IM_{66} and IM_{100} down-regulated the expression of *Mn-SOD, GPx1*, *HSP27* and *HSP70* compared to salmon fed IM_0. In general, these results suggested that replacement of fishmeal with IM in the diets of Atlantic salmon had no effect on the transcription of pro-inflammatory genes in the head kidney cells. There was, however, an effect of dietary IM on the transcription of antioxidant and stress related genes in the leukocytes.

1. Introduction

Finding nutritionally appropriate and sustainable alternatives to fishmeal (FM) and fish oil (FO) for use in aquaculture feeds is an area of intense research, with possible alternative sources of ingredients coming from terrestrial plants, animal by-products, microalgae, macroalgae, mussels or insects, to mention some [1–3]. Insects are part of the natural diet of many fish species and may therefore represent a nutritionally suitable as well as a more sustainable ingredient for future aquafeed production. The black soldier fly (BSF) (*Hermetia illucens*) larvae has emerged as one of the most important candidate species to be used in animal feeds [4,5]. The nutritional composition of BSF larvae has been widely reviewed with emphasis on amino acid composition, lauric acid (C12) content, and potential carrier of essential minerals and

* Corresponding author. Department of Requirement & Welfare, Nordnesgaten 50, 5005, Bergen, Norway.
E-mail address: Ikram.Belghit@hi.no (I. Belghit).

https://doi.org/10.1016/j.fsi.2019.05.042
Received 10 March 2019; Received in revised form 2 May 2019; Accepted 20 May 2019
Available online 21 May 2019

vitamins, with the main conclusion being that BSF larvae can be an optimal feedstuff for several fish species [6–9]. In addition to the suitability of the nutrient content, the larvae of these species have the ability to convert large varieties of food waste (vegetable, fruits, factory waste or animal tissue) into high quality protein and fat mass [10,11]. Today, there is an increase in companies focusing on the farming of these species on a large scale, meaning that insect meal may also be easily available at more competitive prices [12,13].

In their natural environment, many insect larvae, including BSF larvae, live in a harsh environments, such as compost, animal waste (carrion), rotten plant material or manure, which are often infested with many types of microorganisms. The defense strategies of the insects living in these substrates result in the presence of many native bioactive peptides with diverse functional properties, such as anti-microbial, anti-fungal and anti-viral functions [14–16]. Interestingly, dietary intake of insect meal, modulates the gut microbiota of laying hens [17] and was suggested as a potential prebiotic in broiler diets [17]. In addition, the bioactive polysaccharides, silkrose and dipterose isolated from silkmoth (*Antheraea yamamai*) pupae and melon fly (*Bactrocera cucurbitae*) pupae, respectively, have been shown to stimulate the innate immune response in mammalian macrophages [18,19]. Few studies have investigated the effect of dietary insect meals on the immune system in fish species. Dietary inclusion of housefly (*Musca domestica)* at low levels (between, 0.75 and 7.5%) did, however, increase the innate immune system and disease resistance of red sea bream (*Pagrus major*) [20] and black carp (*Mylopharyngodon piceus*) [21]. Furthermore, partial replacement of FM with yellow mealworm (*Tenebrio molitor*) meal, reduced parameters linked to the inflammatory responses in the serum of the European sea bass (*Dicentrarchus labrax*) [22], whereas, it enhanced the innate and adapted immune response and increased the protection against pathogen challenges in yellow catfish (*Pelteobagrus fulvidraco*) [23].

To further investigate the effect of dietary insect meal on the immune system of fish, we performed an *in vitro* experiment using immune cells isolated from Atlantic salmon (*Salmo salar* L.) fed dietary IM [9]. More specifically, the objective was to investigate how a partial or total replacement of dietary FM with BSF meal affect gene response of processes involved in immune-, eicosanoid-, stress- and signaling pathways in isolated head kidney leukocytes before and after exposure to bacterial or viral mimic.

2. Materials and methods

2.1. Diets

The practical details of the feed production and the feeding trial with Atlantic salmon are reported in more detail elsewhere [9]. Briefly, a black soldier fly larvae meal was produced from larvae reared on media partially containing seaweeds (*Ascophyllum nodosum*) mixed with organic waste streams (60:40) (produced by Protix Biosystems BV, Amsterdam, The Netherlands). Post-smolt Atlantic salmon were randomly distributed among 9 sea-cages (n = 3) (5 × 5x5m; 125 m^3; 90 fish per cage) and were fed one of the three isonitrogenous and isolipidic diets (produced by Cargill, Dirdal, Norway) for 8 weeks. The protein sources in the control diet (IM_0) were FM and plant-based proteins (soy protein concentrate, corn gluten meal, wheat gluten meal, pea protein concentrate, 20:80, w/w). The main lipid sources in all the diets were fish oil and rapeseed oil (33:66, w/w). Two experimental diets containing insect were formulated, in which 66% (IM_{66}) and 100% (IM_{100}) of the FM was replaced with IM. The analyzed composition of the experimental diets, including proximate composition, fatty acid composition and total fatty acids are shown in Table 1 (previously published in Belghit et al. [9]).

Table 1
Analyzed composition of the experimental diets (including proximate analysis, fatty acids and total fat composition) fed to Atlantic salmon (*salmo salar*) during 8 weeks before head kidney leukocytes were isolated (Previously published in Belghit et al. [9]).

	IM_0	IM_{66}	IM_{100}
Proximate analysis			
DM (%)	93	94	95
Crude Protein (%)	38	39	39
Crude Lipid (%)	29	29	29
Ash (%)	4.6	4.5	4.5
Carbohydrates (%)	11.6	11.5	11.4
Gross energy (MJ/kg)	24.6	24.8	25.0
TBARS (nmol/g)	3.0	4.2	4.9
***Fatty acids* (g/100g)**			
12:0	< LOQ	1.2	2.3
14:0	2.2	3.2	3.6
16:0	8.5	9.0	9.0
18:0	3.0	3.0	3.0
18:1n-9	40.0	33.0	30.0
18:1n-7	2.5	2.2	2.0
18:2n-6	14.0	12.0	11.0
18:3n-3	6.5	5.6	5.0
18:4n-3	1.4	2.0	2.0
20:4n-6 ARA	0.2	0.2	0.3
20:5n-3 EPA	3.0	4.0	4.4
22:5n-3 DPA	0.3	0.4	0.4
22:6n-3 DHA	2.9	4.0	4.0
Sum saturated FA	15.0	17.0	19.0
Sum MUFA	55.0	52.0	50.0
Sum EPA + DHA	6.0	8.0	8.5
Sum n-3	15.0	16.5	17.0
Sum n-6	14.0	12.3	11.6
Sum PUFA	29.0	29.0	29.0
n-3/n-6	1.1	1.3	1.4
Total FA (mg/g)	265	248	277

IM_0 = diet without insect meal (IM) inclusion; IM_{66} and IM_{100} = 66 and 100% replacement level of FM with IM, respectively. DM = dry matter; TBARS = Thiobarbituric acid-reactive substances; LOQ: limit of quantification (0.01 mg/kg sample). ARA = arachidonic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acid.

2.2. Sampling

Fish were collected at the start of the trial (day 0) and at the end of the feeding trial (day 56). At all samplings, the fish were anesthetized by metocaine (MS222, 0.5 g/10 L), individually weighed and body length measured for growth performance. From 6 fish per cage, liver and viscera were removed and weighed for calculation of organosomatic indices. From additional 2 fish per cage (6 fish/dietary group), the head kidneys were removed and added to a sterile isolation buffer containing 150 mM NaCl and 24 mM EDTA, pH 7.2 for isolation of head kidney leukocytes (n = 6).

2.3. Isolation of head kidney leukocytes

The cells were aspirated with a syringe and then squeezed through a 40 μm Falcon cell strainer. The cells were transferred to 50 mL tubes and washed by centrifugation in a Hettich Zentrifugen, 320R, at 400 g, 5 min, and 4 °C. Cell pellets were re-suspended in the isolation buffer and layered carefully on top of equal amounts of diluted Percoll in densities of 1.08 g/mL and 1.06 g/mL. The tubes were centrifuged at 600 g, 15 min, at room temperature. The cell layer in the interface was collected and the cells were pelleted by centrifugation, 400*g*, 4 °C, and 5 min. An additional washing step in the isolation buffer was performed before the viability of the isolated cells was assessed. The viability of the head kidney cells was above 85%.

2.4. Cell medium and reagent

Leibovits-15 (L-15, Sigma) medium was supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, cat#14–801F), 1% glutamax (Gibco, cat#35056), 1% Antibiotic Antimycotic (Penicillin-Streptomycin 50U/mL, Bio- Whittaker, cat#17-602E), and was designated as a complete medium, cL-15. Lipopolysaccharide from *Pseudomonas aeruginosa* (LPS, cat# L-7018) and polyinosinic: polycytidylic acid (poly I: C, cat# P9582) were purchased from Sigma-Aldrich.

2.5. Cell cultures

1×10^7 of leukocytes were seeded into each well of 6 well culture plates (Costar, cat#3335, Sigma-Aldrich) and cL-15 medium was added to a final volume of 2 mL. The cultures were incubated for 24 h in the dark in a normal atmosphere incubator (Sanyo Incubator) at 9 °C. The second day of culturing some of the cells received 100 μg/mL LPS or 50 μg/mL poly I: C. Untreated cultures were included as controls (Ctl). All cultures were incubated for an additional 24 h in the incubator device before sampled for analysis. The cell suspensions were centrifuged and the pelleted cells were homogenized in 600 μl RTL-Plus buffer (RNeasy®Plus kit Qiagen) using a syringe and frozen at −80 °C, for later RNA extraction.

2.6. RNA extraction

Total RNA was extracted from head kidney leukocytes using RNeasy Plus Mini kit (Qiagen) according to the manufacturer's protocol; RNeasy Plus mini Handbook. The quantity and quality of RNA were assessed using the NanoDrop ND-1000 UV Spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity was assessed using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA). The samples used in this experiment had 260/280 nm absorbance ratios of 2.0 ± 0.1 and 260/230 nm ratios of 2.4 ± 0.1 (mean ± STDEV) and RIN-values superior to 8.0 indicating RNA samples isolated from head kidney leukocytes suitable for RT-qPCR.

2.7. Quantitative real time RT- PCR (qPCR)

A two-step qPCR protocol was used to measure the mRNA levels of the target genes in salmon head kidney leukocytes. The PCR primer sequences, GenBank accession numbers and PCR efficiencies used, are shown in Table 2. Briefly, the reverse transcription reactions were run in duplicate on 96 well reaction plates with the GeneAmp PCR 9700 machine (Applied Biosystems, Foster City, Ca, USA), using TaqMan reverse transcription reagent containing Multiscribe Reverse Transcriptase (50 U/μL) (N808-0234, Applied Biosystems). For efficiency calculations, a standard dilution curve was made, using four serial dilutions (500-63 ng total RNA) in triplicates. A total of 250 ng RNA was added to the reaction for each sample. No template control (ntc) and no amplification control (sample devoid of RT enzyme) (nac) were run for quality assessment. The RT reaction was performed with a GeneAmp PCR System 9700 machine by the following program; an incubation step for 10 min at 25 °C, continuing with RT reaction at 4 °C for 60 min by using oligo dTprimers (2.5 mM) in 50 μL total volume, and finally the inactivation in 5 min at 95 °C. Gene expression was quantified with qPCR on the Lightcycler 480 (Roche Applied Sciences, Basel Switzerland) by the following program; 5 min activation and denaturizing step at 95 °C followed by 45 cycles of 10s denaturizing step at 95 °C, 10s annealing step at 60 °C and a 10s synthesis step at 72 °C, followed by a melt curve analysis and cooling at 4 °C. The stability of reference genes was calculated by the program geNorm version 3.5. The two reference genes Elongation factor 1α and β-actin were used to calculate the mean normalized expression (MNE) for the target genes (M < 1.5).

2.8. Statistics

All statistical analyses were performed using the free software environment R (R Development Core Team, 2011). The experiment was designed to use a 2x2-way factorial ANOVA design with diets fed to the salmon prior to cell isolation (IM_0, IM_{66} and IM_{100}%) and treatment of the isolated cells (Ctl, LPS and poly I: C) as the two varying factors. Differences due to dietary treatments or to the cell culture treatments of the isolated cells were detected by two-way ANOVA and Tukey's post hoc test using the packages *nlme* [24] and *multcomp* [25]. All data were tested for homogeneity of variance by Levene's test. Data, which were identified as non-homogeneous, were subjected to a non-parametric analysis (Kruskal Wallis test). Data are presented as mean with standard deviation (SD) and a significance level of 95% was used ($P \leq 0.05$). Figures were made using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Effects of dietary treatments on growth performances in Atlantic salmon

At start of the trial, the fish had a mean weight of 1398 ± 0.03 g. After 8 weeks, the fish had grown to approximatively two fold of the initial body weight (2550 ± 0.04 g) (Table 3). There were no significant effects of IM inclusion on final weight or any of the growth or feed intake parameters (specific growth rate (SGR), food conversion ratio (FCR), feed intake (FI), hepatic somatic index (HSI) and visceral somatic index (VSI), Table 3).

3.2. Effects of dietary treatments and immune stimulation on genes involved in the inflammatory response in isolated salmon head kidney leukocytes

The transcription of the inflammatory related genes interleukin 1 β (*IL-1β)*, *IL-8, IL-10* and tumor necrosis factor α (*TNF-α)* was strongly induced by LPS compared to the non-treated cells (Ctl) in head kidney leukocytes isolated from Atlantic salmon fed dietary IM_0, IM_{66} and IM_{100} (two-way ANOVA, $P < 0.0001$) (Fig. 1a–d). The experimental diets used prior to isolation of head kidney cells had no significant effect on the expression of these genes and no significant interactions between the pre-fed diets and the treatments of the cells were present (Fig. 1a–d).

Exposure of head kidney leukocytes (isolated from salmon fed IM_0 and IM_{66}) to poly I: C led to a significant up-regulation of toll-like receptor 3 (*TLR3)* compared to the non-treated cells (Ctl) ($P = 0.002$) (Fig. 1e). There were no significant effects of the pre-fed dietary replacement of FM with IM on the transcription of *TLR3* in head kidney cells (Fig. 1e).

The transcription of *TLR22* was significantly lower in poly I: C and LPS treated cells than Ctl leukocytes isolated from salmon fed IM_{66} and IM_{100} ($P < 0.001$) (Fig. 1f). Leukocytes challenged with LPS and poly I: C isolated from salmon fed IM_0 did not show any significant difference compared to the Ctl group (Fig. 1f). The diets had a significant effect on the expression of *TLR22*; head kidney leukocytes isolated from fish fed IM_{66} and IM_{100} had a lower expression of *TLR22* than leukocytes isolated from salmon fed IM_0 in LPS treated cells ($P = 0.001$) (Fig. 1f). Head kidney leukocytes isolated from salmon, all diet group, transcribed the same level of *TLR9* (data not shown).

3.3. Effects of dietary treatments and immune stimulation on genes involved in signaling pathway and eicosanoid synthesis in isolated salmon head kidney leukocytes

The transcription of the transcription factor, *ccaat*-enhancer-binding

Table 2
QPCR primer sequences, GenBank accession numbers and amplification efficiencies of salmon head kidney leukocytes.

Primers	Forward	Reverse	Accession number	Amplification efficiency
IL-1 β	*GTATCCCATCACCCCATCAC*	GCAAGAAGTTGAGCAGGTCC	NM_001123582	2.1
IL-10	GGCTTCCCTGTTGGACGAAG	TCAGTGTTTGCGCCTCTTAG	XM_014186180.1	2.2
IN-8	GAGCGGTCAGGAGATTTGTC	TTGGCCAGCATCTTCTCAAT	NM_001140710	2.2
TNF-α	GGCGAGCATACCACTCCTCT	TCGGACTCAGCATCACCGTA	AY848945	2.0
TLR22	AAAGGATGAGGACCCGATG	GCCAACCTCTCCCTGCTAC	NM_001124412	2.1
TLR3	GTTTCATGGTCAATTACAGTAGG	TGGTTAATGAGTGCAATAGTGG	CB499949	2.1
TLR9	ATAGTGGCCGCCAAAGATCC	ACATGAACAGCTGCCGTGTA	NM_001123653.1	2.2
C/EBPβ	CGC GTG GAG CAG CTG TCA AGA	TGG GCA CTC CGG TGT GGC TA	NM_001139913	2.1
P38MAPK	GGCACACAGACGATGAGATG	ACAGCGTTCTGCCAGTGAG	EF123661	2.2
Cd36	GGATGAACTCCCTGCATGTGA	TGAGGCCAAAGTACTCGTCGA	AY606034	2.1
LOX5	ACT AAG TTT GCT GCT TCG G	CTG ACT CCA GAC CTC GTG	CA387866	2.1
COX2	GGAGGCCTACTCCAACCTATT	CGAACATGAGATTGGAACC	AY848944	2.3
PTGES	TCC AGC CAA TGT CTT AGT	AAG CAC GGT ATA ACT GAAC	LOC106571940	2.0
PTGDS	ATCCCAGGCCGCTTCAC	ACACGCATGTCATTTTCATTGTT	LOC100196042	2.0
GPx1	TCTCCTGCCATAACGCTTGA	GTGATGAGCCCATGGCCTTA	EH033571	2.1
Mn-SOD	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	DY718412	2.0
Cu/Zn-SOD	GAAGCTGACGGGAGAGATCG	GAGTTCGGGGGTAAGCTACG	BG936553	2.2
HSP 70	CCCCTGTCCCTGGGTATTG	CACCAGGCTGGTTGTCTGAGT	BG933934	2.1
HSP 27	GCACATGGGCCTCTGACTAT	AACAGTGAGTGGCCGTAACA	XM_014136598	2.1
B-actin	CCA AAG CCA ACA GGG AGAA	AGG GAC AAC ACTGCC TGG AT	BG933897	2.1
EIF1α	TGCCCCTCCAGGATGTCTAC	CAGCGTGATAGACTCGTTGC	AF321836	2.0

Table 3
Mean growth performance and feed utilization of Atlantic salmon fed diets with increasing replacement of fish meal with insect meal during 8 weeks.

	IM_0	IM_{66}	IM_{100}
IW (g)	1398 ± 0.03	1386 ± 0.04	1409 ± 0.02
FW (g)	2552 ± 0.03	2518 ± 0.04	2535 ± 0.05
SGR	1.11 ± 0.06	1.11 ± 0.04	1.08 ± 0.01
FCR	1.11 ± 0.02	1.13 ± 0.03	1.13 ± 0.02
FI	2.11 ± 0.09	2.11 ± 0.02	2.09 ± 0.06
HSI	1.17 ± 0.21	1.19 ± 0.10	1.31 ± 0.13
VSI	10.9 ± 0.9	10.6 ± 1.0	11.2 ± 0.7

IM_0 = diet without insect meal (IM) inclusion; IM_{66} and IM_{100} = 66 and 100% replacement level of FM with IM, respectively; IW = initial weight; FW = final weight; SGR = specific growth rate (100 x [ln final body weight (g) - ln initial body weight (g)]/days); FCR = food conversion ratio (feed intake (g)/fish weight gain (g)); FI = feed intake (quantity of food taken/days); HSI = Hepatic Somatic Index (liver weight (g)/body weight (g) * 100); VSI = Visceral Somatic Index (viscera weight (g)/body weight (g) * 100). Data are presented as means ± SD (n = 3). No significant differences ($P \leq 0.05$, one-way ANOVA) were recorded among the dietary groups.

proteins (*C/EBPβ*), was significantly affected by both the cell treatments and the experimental diets used prior to the cell isolation ($P < 0.001$) (Fig. 2a). The transcription of *C/EBPβ* was significantly down-regulated in leukocytes isolated from salmon pre-fed the three experimental diets (IM_0, IM_{66} and IM_{100}) stimulated with poly I: C as compared to the Ctl leukocytes ($P < 0.001$) (Fig. 2a). In addition, head kidney leukocytes isolated from fish pre-fed with IM_{66} and IM_{100} had a lower *C/EBPβ* expression than cells isolated from fish pre-fed IM_0 in LPS and Ctl leukocytes ($P < 0.001$) (Fig. 2a).

Head kidney leukocytes isolated from salmon fed all diets (IM_0, IM_{66} and IM_{100}) and challenged with LPS and poly I: C had a significantly lower transcription of mitogen activated phosphokinase p38 (*p38MAPK*) compared to untreated cells (Ctl) ($P < 0.001$). Additionally, head kidney cells, untreated (Ctl) and challenged with LPS and poly I: C, showed a significantly lower expression of *p38MAPK* when isolated from fish pre-fed with increasing inclusion of IM (IM_{66} and IM_{100}) compared to salmon pre-fed with FM as a protein source (IM_0) ($P < 0.001$) (Fig. 2b).

Head kidney leukocytes isolated for all dietary groups (IM_0, IM_{66} and IM_{100}) and challenged with LPS had a higher expression of fatty acid translocase protein (*cd36*) compared to untreated cells (Ctl) ($P < 0.001$) (Fig. 2c). No differences were recorded due to the feeding trial prior to isolation of head kidney cells and no significant interactions between the diets and the cell treatments were observed (Fig. 2c).

The transcription of cyclooxygenase 2 (*COX2*) was strongly induced by LPS compared to the non-treated cells (Ctl) in head kidney leukocytes isolated from Atlantic salmon fed all dietary groups ($P < 0.001$) (Fig. 3a). LPS and poly I: C down-regulated lipoxygenase-5 *(LOX5*) transcription in head kidney leukocytes isolated from salmon, all dietary groups, compared to Ctl group ($P < 0.001$) (Fig. 3b). In addition, leukocytes isolated from fish pre-fed insect based diets (IM_{66} and IM_{100}) had significantly lower expression of *LOX5* when compared to cells isolated from salmon pre-fed diet devoid of IM (IM_0) in LPS challenged leukocytes ($P < 0.001$) (Fig. 3b). Neither diets fed prior to isolation of head kidney cells nor LPS or poly I:C treated leukocytes influenced prostaglandin D synthase (*PTGDS)* and prostaglandin E synthase (*PTGES*) transcription in the head kidney leukocytes (Fig. 3c–d).

3.4. Effects of dietary treatments and immune stimulation on genes involved in stress response in isolated salmon head kidney leukocytes

The transcription of all stress-related gene expression investigated in the present study were significantly affected by both treatments and diets in the head kidney leukocytes isolated from Atlantic salmon. No significant interactions were present between cell treatments and diets used prior to cell isolation for those expressed genes. Glutathione peroxidase enzyme (*GPx1*), copper/zinc-superoxide dismutase (*Cu/Zn-SOD*) and manganese-SOD (*Mn-SOD)* transcription were significantly ($P < 0.001$, $P = 0.03$ and $P < 0.001$, respectively) down-regulated in poly I: C treated head kidney leukocytes compared to Ctl and LPS treated cells isolated from salmon pre-fed IM_0 and IM_{66} (*GPx1*, *Cu/Zn-SOD* and *Mn-SOD*) and IM_{100} (*GPx1* and *Cu/Zn-SOD*) (Fig. 4a–c). Furthermore, head kidney leukocytes isolated from salmon fed the insect-based diets (IM_{66} and IM_{100}) prior to isolation of head kidney cells showed significantly lower *GPx1, Cu/Zn-SOD* and *Mn-SOD* ($P = 0.001$, $P = 0.05$ and $P = 0.01$, respectively) expression compared to leukocytes isolated from salmon pre-fed IM_0, in LPS challenged cells (*GPx1, Cu/Zn-SOD* and *Mn-SOD*) and non-challenged leukocytes (Ctl) (*GPx1)* (Fig. 4a–c).

Heat shock proteins 27 and 70 (*HSP27* and *HSP70,* respectively) transcription were significantly down-regulated in leukocytes isolated from salmon pre-fed IM_{66} and IM_{100} (*HSP70)* and IM_0 and IM_{66} (*HSP27*), challenged with poly I: C compared to Ctl cells, but not

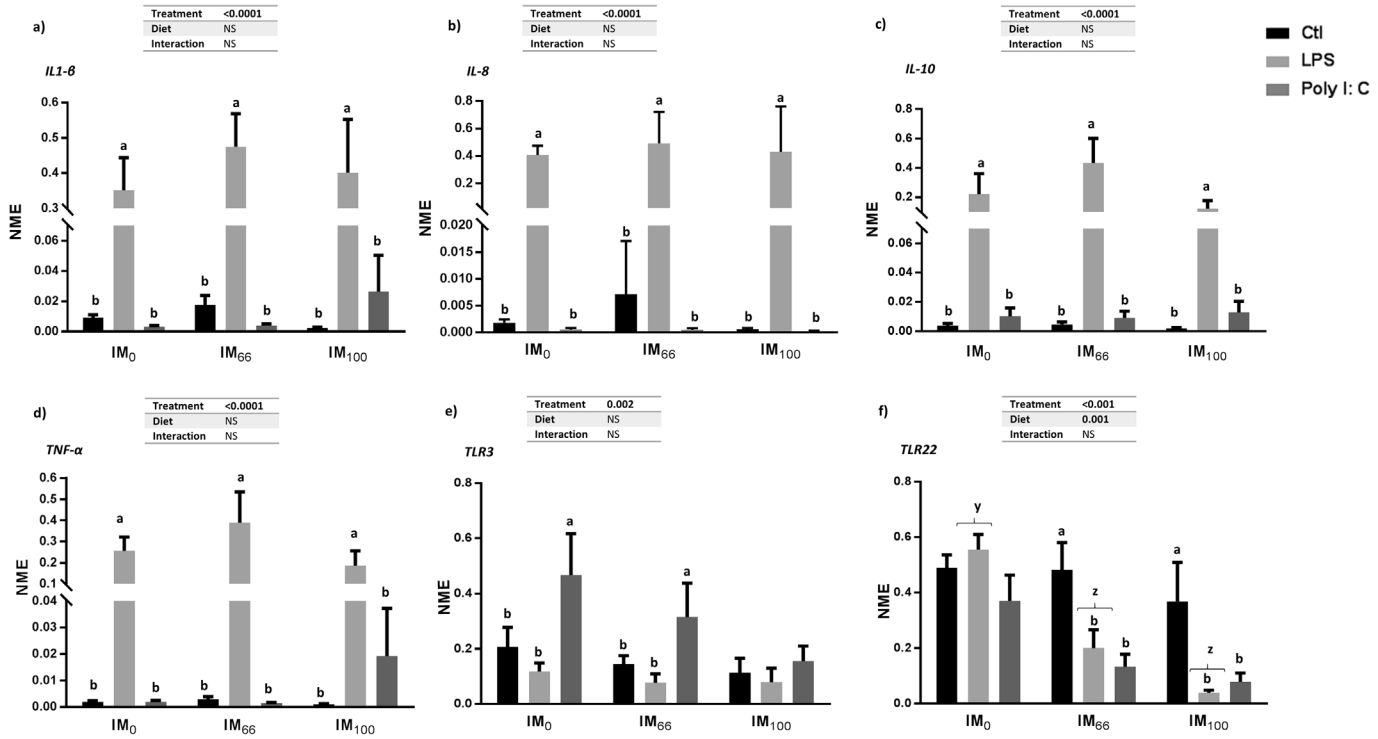

Fig. 1. Effects of dietary treatments and immune stimulation on genes involved in the inflammatory response in salmon head kidney leukocytes. qPCR analysis of; a) *IL1-β*, b) *IL-8*, c) *IL-10*, d) *TNF-α*, e) *TLR3* and f) *TLR22* gene expression in leukocytes isolated from head kidney of Atlantic salmon pre-fed IM_0, IM_{66} and IM_{100} diets, before (Ctl) and after stimulation with LPS (100 μg/mL) and poly I: C (50 μg/mL). IM_0 = diet without insect meal (IM) inclusion; IM_{66} and IM_{100} = 66 and 100% replacement level of FM with IM, respectively. Values are means (n = 6), with their standard deviation represented by vertical bars. 2x2-way factorial ANOVA design with treatment (Ctl, LPS and poly I: C) and inclusion of IM in the diets (IM_0, IM_{66} and IM_{100}%) as varying factors and interaction between the main effects of the two factors (treatment × diet) ($P \leq 0.05$, two-way ANOVA, followed by the Student–Newman–Keuls multiple-comparison test). $^{a, b}$; significant effect of treatment ($P \leq 0.05$, two-way ANOVA). $^{y, z}$; significant effect of IM inclusion in the diet ($P \leq 0.05$, two-way ANOVA). No significant interaction between treatment and IM inclusion were recorded among the dietary groups. NME; normalized mean expression.

significantly different than LPS treated cells (P = 0.02 and 0.03, respectively) (Fig. 4d–e). Moreover, the transcription of these two genes, significantly decreased in leukocytes challenged with LPS and isolated from salmon pre-fed dietary insect meal (IM_{66}, IM_{100}) compared to those isolated from fish pre-fed IM_0 (P = 0.04) (Fig. 4d–e).

4. Discussion

In summary, the main results of the current study showed that a partial or total replacement of fish meal with IM in the diet of Atlantic salmon did not influence the inflammatory transcription genes in the isolated head kidney leukocytes treated with LPS or poly I: C. However, head kidney cells isolated from salmon fed with graded inclusion level of IM and treated with LPS, down-regulated the expression of *TLR22, C/EBPβ, p38MAPK and LOX5* transcripts. Furthermore, leukocytes isolated from fish fed with dietary IM, down-regulated the expression of oxidative stress genes, indicating probably that insect-based diets affect the cellular stress response in salmon head kidney under a bacterial challenge.

The results from the present study showed that head kidney leukocytes isolated from all diet groups responded strongly to LPS with increased transcription of the pro-inflammatory *IL-1β*, *IL-8*, *TNF-α*, and the anti-inflammatory *IL-10* cytokines. These results confirmed the suitability of the *in vitro* model used in the current experiment, as the first line of cellular innate immune defense of fish recognize pathogen-associated molecular patterns (PAMPs), such as LPS or poly I: C, by pathogen recognition receptors (PRRs), including TLR and ILs [26,27]. The results showed that LPS and poly I: C induced different transcriptional genes response in salmon head kidney cells, in line with previously observed responses in fish head kidney cells [27–29]. Poly I: C, a synthetic dsRNA, is capable of eliciting antiviral immunity responses via its recognition by TLR3. In the current study, the expression of *TLR3* was induced following exposure to poly I: C in leukocytes isolated from salmon pre-fed IM_0 and IM_{66}. Similar results were obtained in different fish species, where *TLR3* transcript was up-regulated after a viral mimic, in *in vivo* or *in vitro* models [27,29–31]. However, in the present study, the expression of this gene was down-regulated in poly I: C treated leukocytes isolated from fish pre-fed with the highest dietary IM inclusion (IM_{100}). These results suggested that a complete replacement of FM with IM in the diet of salmon might modulate the viral response of head kidney cells. This is, however, only based on transcription of *TLR3* and further investigations of the mechanism underlying the relationship between poly I:C,TLRs and other virus induced markers are needed.

Furthermore, some studies have shown that the expression of the fish-specific *TLR22* is up-regulated following a viral or bacterial stimulations in systemic immune organs and cells of different fish species, including common carp (*cyprinus carpio*), Atlantic cod (*Gadus morhua*) and rainbow trout (*oncorhynchus mykiss*) [32–34]. However, other studies demonstrated that the expression of this gene is down-regulated in kidney and blood after a viral challenge in rohu (*Labeo rohita*) [35]. In the current study, the expression of *TLR22* was down-regulated in LPS and poly I:C treated leukocytes isolated from salmon pre-fed insect-based diets compared to fish fed the control diet (IM_0). These results implied probably different role of *TLR22* in different tissues and varied among fish species. In addition to *TLR22* transcript, the transcription of the transcription factor, *C/EBPβ*, decreased in a linear manner with increasing IM in the diet of Atlantic salmon in LPS treated leukocytes. From this, we infer that the replacement of FM with IM in the diet of salmon might have affected LPS induced pathways involved in TLR22

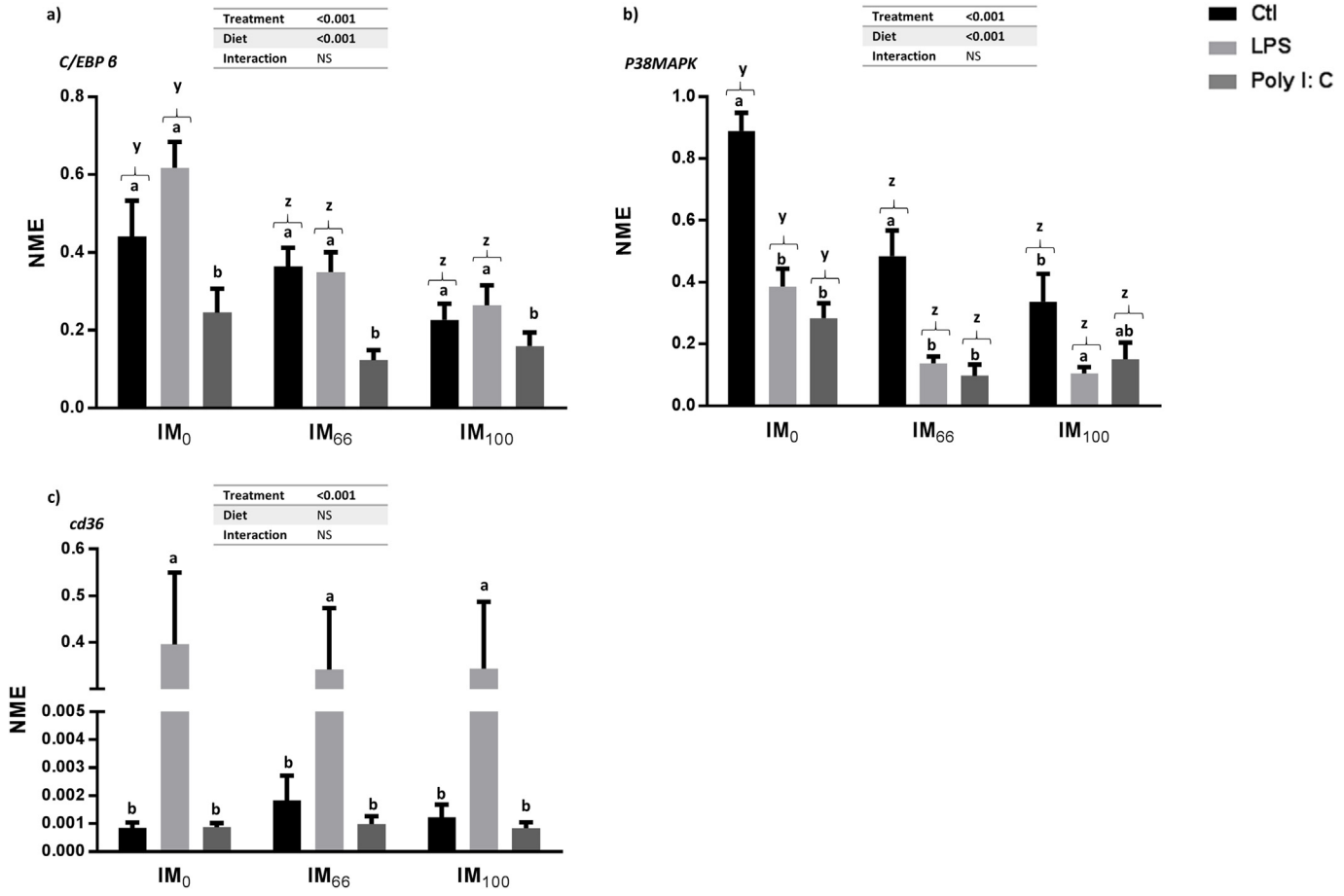

Fig. 2. Effects of dietary treatments and immune stimulation on genes involved in signaling pathway in salmon head kidney leukocytes. qPCR analysis of; a) *C/EBP β*, b) *P38MAPK* and c) *cd36* gene expression in leukocytes isolated from head kidney of Atlantic salmon pre-fed IM_0, IM_{66} and IM_{100} diets, before (Ctl) and after stimulation with LPS (100 μg/mL) and poly I: C (50 μg/mL). IM_0 = diet without insect meal (IM) inclusion; IM_{66} and IM_{100} = 66 and 100% replacement level of FM with IM, respectively. Values are means (n = 6), with their standard deviation represented by vertical bars. 2x2-way factorial ANOVA design with treatment (Ctl, LPS and poly I: C) and inclusion of IM in the diets (IM_0, IM_{66} and IM_{100}%) as varying factors and interaction between the main effects of the two factors (treatment × diet) ($P \leq 0.05$, two-way ANOVA, followed by the Student–Newman–Keuls multiple-comparison test). $^{a, b}$; significant effect of treatment ($P \leq 0.05$, two-way ANOVA). $^{y, z}$; significant effect of IM inclusion in the diet ($P \leq 0.05$, two-way ANOVA). No significant interaction between treatment and IM inclusion were recorded among the dietary groups. NME; normalized mean expression.

and *C/EBPβ* signaling. Some recent studies investigated the potential effect of yellow mealworm meal and housefly meal on disease resistance of different fish species challenged with bacterial pathogens [21–23]. Su et al. [23], also found an increase in the innate and the adaptative immune response in yellow catfish (*Pelteobagrus fulvidraco*) (before and after the bacterial challenge), while inclusion of mealworm in the diet of European seabass (*Dicentrarchus labrax*), resulted in a strong reduction of parameters linked to the anti-inflammatory activity [22]. In the feeding trial used for the isolation of head kidney cells in the current trial, no differences in growth performances or feed utilization were observed due to the dietary treatments (Table 3). Thus, it can be assumed that the fish of all dietary groups were provided with adequate amounts of essential nutrients. Dietary insects might contain diverse components with beneficial effects, such as chitin. This polymer acts as PAMPs by binding to PRRs and thus stimulating the production of many cytokines and immune mediators. As such, the chitin from the insect-based diets of the current trial should have the potential to stimulate the innate immune response, as shown in both mammals [36,37] and fish [38–40]. Inclusion of crustacean chitin (< 1%) in the diet of gilthead seabream (*Sparus aurata*) increased head-kidney leucocyte respiratory burst, phagocytic and cytotoxic activities [40]. In the present experiment, however, pre-feeding salmon with dietary insect meal attenuated the expression of *TLR22* and *C/EBPβ* in isolated head kidney leukocytes challenged with the bacterial mimic. It has been estimated that BSF meals contains between 3 and 5% chitin [41], although the content of dietary chitin has not been measured in the current trial. Therefore, the reduced expression of *TLR22* and *C/EBPβ* in the leukocytes of fish pre-fed IM might be due to the content of chitin or due to other immune-modulating components in BSF larvae meal. Further studies should be carried out to understand the effects of dietary insect chitin on the immune functions in fish species.

Previously, Holen et al. [42] demonstrated that *p38MAPK* gene, a transducer of extracellular stress and inflammatory signals, was down-regulated in head kidney leukocytes exposed to LPS, due to activation of the p38 protein or signals upstream leading to a negative feedback loop of regulation [43,44]. Similarly, in the present study, exposure of head kidney leukocytes to LPS but also poly I: C, from all diet groups, down-regulated *p38MAPK* transcription. On the other hand, in the current study, the expression of *cd36* transcript was highly induced in the leukocytes treated with LPS compared to poly I: C or untreated cells isolated from all the dietary salmon groups. In mammals, the scavenger receptor CD36, plays an important role in LPS-induced inflammation process via activation of down-stream signaling cascade to modulate the production of cytokines and interaction with TLR4 [45]. Holen et al. [44] found that the expression of the transcript *cd36* was highly expressed in head kidney leukocytes isolated from salmon pre-fed soybean oil diet compared to cells isolated from salmon pre-fed palm-, linseed-, rapeseed- or fish-oil sources. However, the expression of this gene was

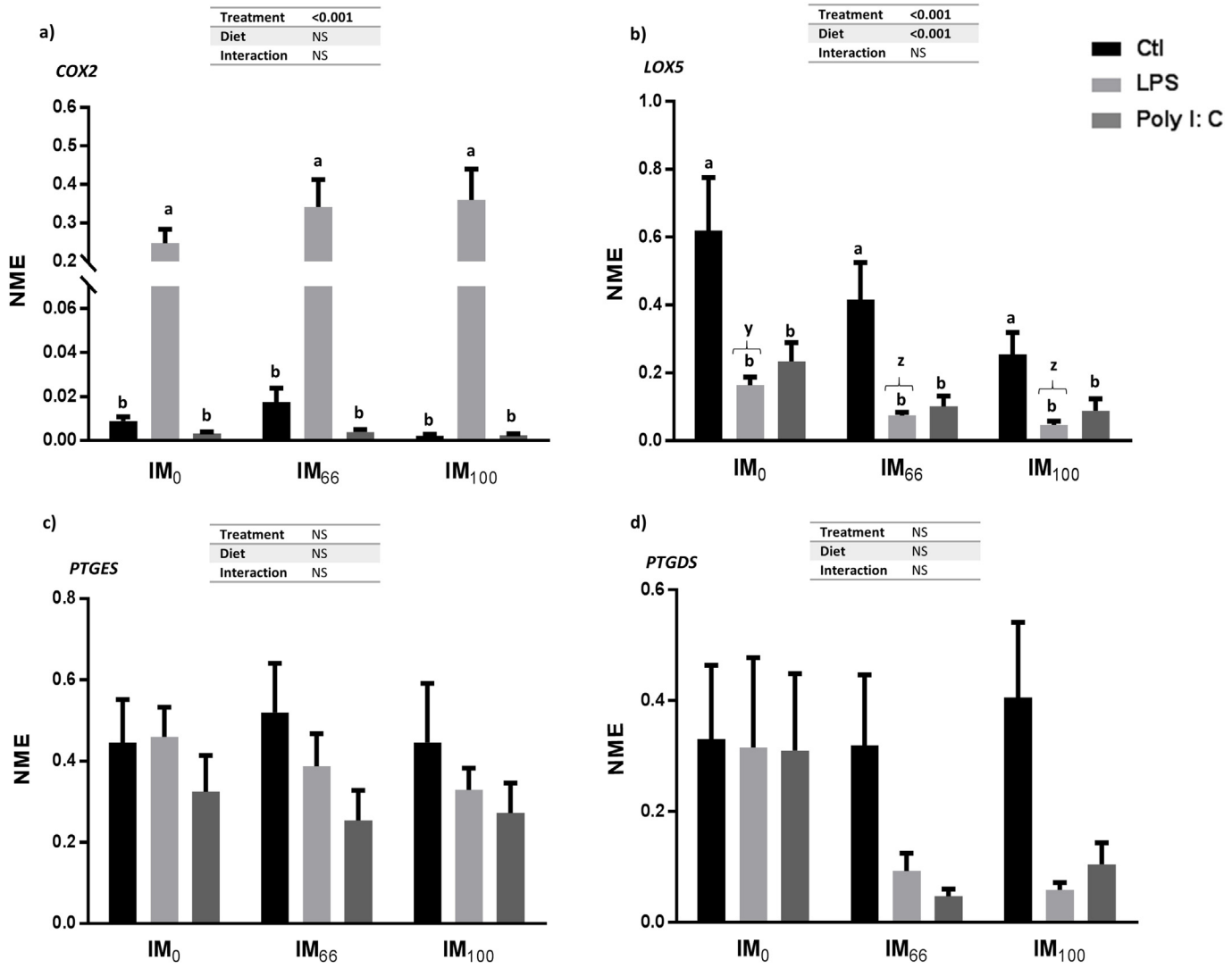

Fig. 3. Effects of dietary treatments and immune stimulation on genes involved in eicosanoid synthesis in salmon head kidney leukocytes. qPCR analysis of; a) *COX2*, b) *LOX5*, c) *PTGES* and d) *PTGDS* gene expression in leukocytes isolated from head kidney of Atlantic salmon pre-fed IM_0, IM_{66} and IM_{100} diets, before (Ctl) and after stimulation with LPS (100 μg/mL) and poly I: C (50 μg/mL). IM_0 = diet without insect meal (IM) inclusion; IM_{66} and IM_{100} = 66 and 100% replacement level of FM with IM, respectively. Values are means (n = 6), with their standard deviation represented by vertical bars. 2x2-way factorial ANOVA design with treatment (Ctl, LPS and poly I: C) and inclusion of IM in the diets (IM_0, IM_{66} and IM_{100}%) as varying factors and interaction between the main effects of the two factors (treatment × diet) ($P \leq 0.05$, two-way ANOVA, followed by the Student–Newman–Keuls multiple-comparison test). $^{a, b}$; significant effect of treatment ($P \leq 0.05$, two-way ANOVA). $^{y, z}$; significant effect of IM inclusion in the diet ($P \leq 0.05$, two-way ANOVA). No significant interaction between treatment and IM inclusion were recorded among the dietary groups. NME; normalized mean expression.

not affected by the dietary treatments prior to leukocytes isolation in the present study.

The enzymes LOX5 and COX2 are responsible for the biosynthesis of inflammatory mediators (such as leukotrienes and prostaglandins) from arachidonic acid (ARA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) [46]. The transcription of *COX2* was up-regulated in isolated leukocytes treated with LPS, whereas, *LOX5* gene transcription was down-regulated in salmon leukocytes treated with LPS or poly I: C, isolated from all the dietary groups. EPA, ARA and DHA are able to promote or suppress inflammatory and immune response, by affecting the membrane fluidity of the cells. Interestingly, in the current study, head kidney leukocytes isolated from salmon pre-fed dietary insect meal had a decreased expression of *LOX5* compared to cells isolated from salmon pre-fed FM and plant proteins, after an exposure to LPS. Holen et al. [44], demonstrated that LPS down-regulated *LOX5* expression in head kidney cells of salmon fed vegetable oil diets. In this study, the authors suggested that the n-3/n-6 ratio might impact the eicosanoid production in the head kidney isolated from salmon, and showed that *PGDS* and *PGES* transcripts were significantly up-regulated in leukocytes isolated from salmon fed with soybean oil diet compared to palm oil and rapeseed oil diets [44,47]. In our trial, the experimental diets and the whole body content of EPA, DHA, total n-3 and n-3/n-6 ratio increased in the salmon fed with increasing inclusion of dietary IM, while the total n-6 content decreased in fish fed IM [9]. Therefore, the fatty acids composition of the diets might affected the membranes of head kidney leukocytes and thus the functioning of these cells as shown in head kidney cells exposed to different FAs (EPA, DHA and ARA) [47] or isolated from salmon fed varying oil sources [44]. The particularity of the fatty acid profile of BSF larvae is its high concentration of the medium chain fatty acid (MC-FA) lauric acid (LA, C12) [48,49]. MC-FA are FA with carbon chains of 6–12 carbon, and have been widely investigated due to their rapid oxidation and absorption, but also their antimicrobial and antiviral properties in gut microbiota of farmed animals [50,51]. Recently, an experimental trial with different inclusion levels of the MC-FA-rich BSF larvae, were conducted in piglets [52]. In this study, supplementation of the culture media with LA

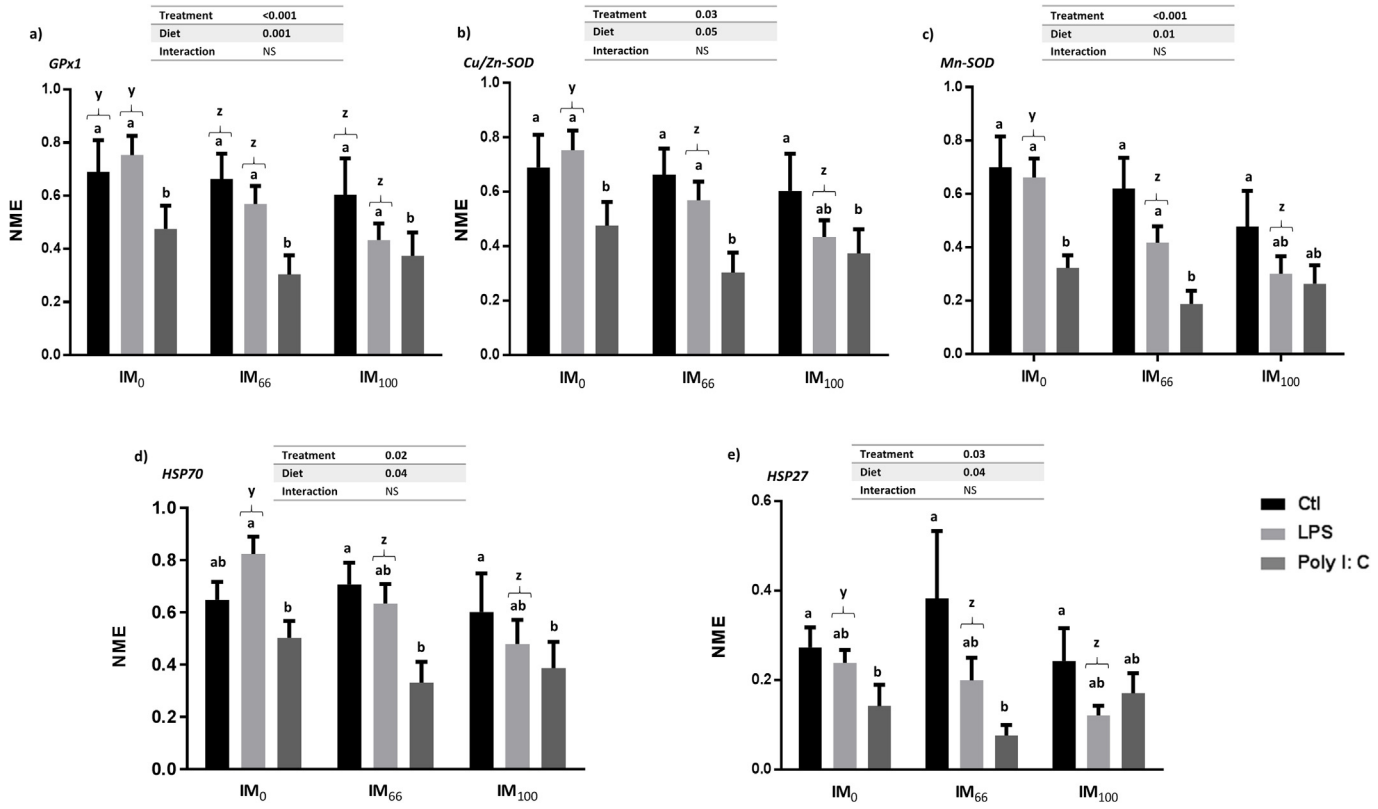

Fig. 4. Effects of dietary treatments and immune stimulation on genes involved in stress response in salmon head kidney leukocytes. qPCR analysis of; a) *GPx1*, b) *Cu/Zn-SOD*, c) *Mn-SOD*, d) *HSP70* and e) *HSP27* gene expression in leukocytes isolated from head kidney of Atlantic salmon pre-fed IM_0, IM_{66} and IM_{100} diets, before (Ctl) and after stimulation with LPS (100 μg/mL) and poly I: C (50 μg/mL). IM_0 = diet without insect meal (IM) inclusion; IM_{66} and IM_{100} = 66 and 100% replacement level of FM with IM, respectively. Values are means (n = 6), with their standard deviation represented by vertical bars. 2x2-way factorial ANOVA design with treatment (Ctl, LPS and poly I: C) and inclusion of IM in the diets (IM_0, IM_{66} and IM_{100}%) as varying factors and interaction between the main effects of the two factors (treatment × diet) ($P \leq 0.05$, two-way ANOVA, followed by the Student–Newman–Keuls multiple-comparison test). $^{a, b}$; significant effect of treatment ($P \leq 0.05$, two-way ANOVA). $^{y, z}$; significant effect of IM inclusion in the diet ($P \leq 0.05$, two-way ANOVA). No significant interaction between treatment and IM inclusion were recorded among the dietary groups. NME; normalized mean expression.

suppressed growth of lactobacilli, with antibacterial effects against D-streptococci infections in piglet. It has also been shown that LA stimulate the expression of inflammatory-related genes, *IL-1α*, *TLR*, inducible nitric oxide synthase (*iNOS*), and *COX2*, in mammalian cells [36]. However, as seen for *TLR22, C/EBPβ* and *p38MAPK,* the transcription of *LOX5* was also down-regulated in leukocytes isolated from fish pre-fed dietary insect meal containing lauric acid in the current study. Thus, our results suggest that the nutritional composition of insect-based diets might affect the transcription of those genes in the head kidney leukocytes. This is, however, only based on mRNA transcription results in an *in vitro* study, but the effects of dietary insect meal on the innate immunity could be an interesting path to follow further.

Oxidative stress within cells or tissue have profound effects on fish health, and the antioxidant substances that reduce oxidative stress therefore play a significant role as health-benefiting factors. The antioxidant genes, like *SOD*, *catalase*, *GPx1* are easily induced by reactive oxygen species (ROS), and the levels of antioxidant genes can be used to quantify oxidative stress in the cell [53]. The changes in oxidative stress in the fish might be due to a number of processes e.g.; environmental stressors, changes in the metabolism, activity of the immune defense and different levels of contaminants in the diets [54]. Li et al. [55], reported an increase in the expression of a xenobiotic biotransformation enzyme, cytochrome P450 A1 (*Cyp1a1*) in the intestine of salmon fed dietary insects compared to salmon fed FM and soy protein concentrate as protein source. The authors speculate that the higher concentration of certain heavy metals in the IM (such as cadmium) might induce a detoxification process. Although, in the present study, the level of peroxidation product (TBARS) was elevated in the diets containing IM compared to diets devoid of insect ingredients, the values are considered low [56]. In addition, the concentration of heavy metals, pesticides and mycotoxins in the IM diets was not higher than the IM_0 diets [57]. Surprisingly, our results showed that the leukocytes isolated from salmon pre-fed the IM-containing diets (IM_{66} and IM_{100}) in combination with the LPS treatment had a lower gene expression of antioxidant enzymes (*SOD* and *GPx1*) and heat shock protein (*HSP70* and *HSP27*), when compared to cells isolated form salmon pre-fed IM_0. Similar results have been obtained in European Sea bass fed mealworm meal [22]. In this study, the authors found a reduction of stress-related markers, as myeloperoxidase activities and nitric oxide concentration in serum of fish fed with insect larvae compared to fish fed FM. Overall, the reduced expression of genes involved in the oxidative stress after LPS exposure might indicate a reduced ROS production in the cells when the salmon were fed with IM, compared to fish fed with a diet containing FM and plant proteins. In contrast, a down-regulation of the antioxidant genes could also play an aggravating role in the oxidative stress response, if this implies a reduced antioxidant defense system against ROS.

Intensive farming systems open to the marine environment are often exposed to external pathogens, resulting in reduced fish health and welfare. Feed ingredients may provide benefits to the fish, e.g. preventive health care through nutritional means. Indeed, dietary intake of insect meal, modulates the gut microbiota of laying hens [17] and also increase the presence of bacteria of the *carnobacterium* genus in rainbow trout, and thus suggesting insect meal as potential prebiotic in broiler and salmonids diets [17,58]. In addition, insect species contain many native bioactive compounds with antibacterial, antifungal and antiviral

functions [14–16]. In the current study, the effect seen on the reduced expression of the redox response indicated that the experimental diets most likely influenced the head kidney leukocytes after exposure to the bacterial mimic, having an impact on the cellular stress response.

5. Conclusions

In conclusion, our results showed a strong induction of the innate immune transcripts in the head kidney of salmon treated with LPS, but not with poly I: C. Dietary replacement of FM with BSF meal prior to isolation of head kidney cells did not influence the transcription of inflammatory-related genes during the viral or the bacterial stimuli. However, insect-based diets showed indications of having an impact on the cellular stress response in salmon head kidney leukocytes treated with LPS. Thus, based on the current experience and earlier published results on the effect of dietary insect on immune response and diseases resistance in farmed animals, we can conclude dietary IM might contain compounds affecting the health of animals. It is not certain how, or which specific compounds in IM that are bioactive and would affect fish health. Further studies are needed to elucidate the potential beneficial effects of IM in farmed animals.

Acknowledgement

This study was supported by the Norwegian Research Council project Aquafly, grant number 238997 and RAFFPINN, grant number 220634. No potential conflict of interest was reported by the author(s).

References

[1] D.M. Gatlin, F.T. Barrows, P. Brown, K. Dabrowski, T.G. Gaylord, R.W. Hardy, E. Herman, G. Hu, Å. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E.J. Souza, D. Stone, R. Wilson, E. Wurtele, Expanding the utilization of sustainable plant products in aquafeeds: a review, Aquacult. Res. 38 (6) (2007) 551–579.

[2] A.H.L. Wan, S.J. Davies, A. Soler-Vila, R. Fitzgerald, M.P. Johnson, Macroalgae as a sustainable aquafeed ingredient, Rev. Aquacult. 0 (0) (2018).

[3] H.E. Froehlich, C.A. Runge, R.R. Gentry, S.D. Gaines, B.S. Halpern, Comparative terrestrial feed and land use of an aquaculture-dominant world, Proceedings of the National Academy of Sciences, 2018.

[4] J.A. Cammack, J.K. Tomberlin, The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly *Hermetia illucens* (L.) (Diptera: stratiomyidae), Insects 8 (2) (2017) 56.

[5] A. Van Huis, Potential of insects as food and feed in assuring food security, Annu. Rev. Entomol. 58 (1) (2013) 563–583.

[6] E.R. Lock, T. Arsiwalla, R. Waagbø, 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.

[7] I. Belghit, N.S. Liland, R. Waagbø, I. Biancarosa, N. Pelusio, Y. Li, Å. Krogdahl, E.-J. Lock, Potential of insect-based diets for Atlantic salmon (*Salmo salar*), Aquaculture 491 (2018) 72–81.

[8] R. Magalhães, A. Sánchez-López, R.S. Leal, S. Martínez-Llorens, A. Oliva-Teles, 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.

[9] I. Belghit, N.S. Liland, P. Gjesdal, I. Biancarosa, E. Menchetti, Y. Li, R. Waagbø, Å. Krogdahl, E.-J. Lock, Black Soldier Fly Larvae Meal Can Replace Fish Meal in Diets of Sea-Water Phase Atlantic Salmon (*Salmo salar*), Aquaculture, 2019.

[10] C.V. Booram, G.L. Newton, O.M. Hale, R.W. Barker, Dried *Hermetia illucens* larvae meal as a supplement for swine, J. Anim. Sci. 44 (3) (1977) 395–400.

[11] A. Müller, D. Wolf, O. Gutzeit Herwig, The black soldier fly, *Hermetia illucens* – a promising source for sustainable production of proteins, lipids and bioactive substances, Zeitschrift für Naturforschung C (2017) 351.

[12] H.P.S. Makkar, G. Tran, V. Heuzé, P. Ankers, State-of-the-art on use of insects as animal feed, Anim. Feed Sci. Technol. 197 (2014) 1–33.

[13] T.T.X. Nguyen, J.K. Tomberlin, S. Vanlaerhoven, Ability of black soldier fly (Diptera: stratiomyidae) larvae to recycle food waste, Environ. Entomol. 44 (2) (2015) 406–410.

[14] O. Elhag, D. Zhou, Q. Song, A.A. Soomro, M. Cai, L. Zheng, Z. Yu, J. Zhang, Expression Screening, Purification and functional characterization of novel antimicrobial peptide genes from *Hermetia illucens* (L.), PLoS One 12 (1) (2017) e0169582.

[15] S.-I. Park, J.-W. Kim, S.M. Yoe, Purification and characterization of a novel antibacterial peptide from black soldier fly (*Hermetia illucens*) larvae, Dev. Comp. Immunol. 52 (1) (2015) 98–106.

[16] H. Vogel, A. Müller, D.G. Heckel, H. Gutzeit, A. Vilcinskas, Nutritional immunology: diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*, Dev. Comp. Immunol. 78 (2018) 141–148.

[17] L. Borrelli, L. Coretti, L. Dipineto, F. Bovera, F. Menna, L. Chiariotti, A. Nizza, F. Lembo, A. Fioretti, Insect-based diet, a promising nutritional source, modulates gut microbiota composition and SCFAs production in laying hens, Sci. Rep. 7 (1) (2017) 16269.

[18] T. Ohta, A. Ido, K. Kusano, C. Miura, T. Miura, A novel polysaccharide in insects activates the innate immune system in mouse macrophage RAW264 cells, PLoS One 9 (12) (2014) e114823.

[19] T. Ohta, K. Kusano, A. Ido, C. Miura, T. Miura, Silkrose: a novel acidic polysaccharide from the silkmoth that can stimulate the innate immune response, Carbohydr. Polym. 136 (2016) 995–1001.

[20] A. Ido, T. Iwai, K. Ito, T. Ohta, T. Mizushige, T. Kishida, C. Miura, T. Miura, Dietary effects of housefly (*Musca domestica*) (Diptera: muscidae) pupae on the growth performance and the resistance against bacterial pathogen in red sea bream (*Pagrus major*) (Perciformes: sparidae), Appl. Entomol. Zool. 50 (2) (2015) 213–221.

[21] J. Ming, J. Ye, Y. Zhang, X. Yang, C. Wu, X. Shao, P. Liu, The Influence of Maggot Meal and L-Carnitine on Growth, Immunity, Antioxidant Indices and Disease Resistance of Black Carp, *Mylopharyngodon piceus*), 2013.

[22] M.A. Henry, L. Gasco, S. Chatzifotis, G. Piccolo, Does dietary insect meal affect the fish immune system? The case of mealworm, *Tenebrio molitor* on European sea bass, *Dicentrarchus labrax*, Dev. Comp. Immunol. 81 (2018) 204–209.

[23] J. Su, Y. Gong, S. Cao, F. Lu, D. Han, H. Liu, J. Jin, Y. Yang, X. Zhu, S. Xie, Effects of dietary *Tenebrio molitor* meal on the growth performance, immune response and disease resistance of yellow catfish (*Pelteobagrus fulvidraco*), Fish Shellfish Immunol. 69 (2017) 59–66.

[24] J. Pinheiro, D.B.D. S, D.S.R.C. Team, Nlme: Linear and Nonlinear Mixed Effects Models, R Package Version 3, (2010), pp. 1–137.

[25] T. Hothorn, F. Bretz, P. Westfall, Simultaneous inference in general parametric models, Biom. J. 50 (3) (2008) 346–363.

[26] R. Medzhitov, Recognition of microorganisms and activation of the immune response, Nature 449 (2007) 819.

[27] E. Holen, K.K. Lie, P. Araujo, P.A. Olsvik, Pathogen recognition and mechanisms in Atlantic cod (*Gadus morhua*) head kidney cells: bacteria (LPS) and virus (poly I:C) signals through different pathways and affect distinct genes, Fish Shellfish Immunol. 33 (2) (2012) 267–276.

[28] J.K. Chettri, M.K. Raida, L. Holten-Andersen, P.W. Kania, K. Buchmann, PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*), Dev. Comp. Immunol. 35 (4) (2011) 476–482.

[29] G.P. Martins, M. Espe, Z. Zhang, I.G. Guimarães, E. Holen, Surplus arginine reduced lipopolysaccharide induced transcription of proinflammatory genes in Atlantic salmon head kidney cells, Fish Shellfish Immunol. 86 (2019) 1130–1138.

[30] C. Fierro-Castro, L. Barrioluengo, P. López-Fierro, B.E. Razquin, A.J. Villena, Fish cell cultures as *in vitro* models of inflammatory responses elicited by immunostimulants. Expression of regulatory genes of the innate immune response, Fish Shellfish Immunol. 35 (3) (2013) 979–987.

[31] A. Caballero-Solares, J.R. Hall, X. Xue, K. Eslamloo, R.G. Taylor, C.C. Parrish, M.L. Rise, The dietary replacement of marine ingredients by terrestrial animal and plant alternatives modulates the antiviral immune response of Atlantic salmon (*Salmo salar*), Fish Shellfish Immunol. 64 (2017) 24–38.

[32] H. Li, G. Yang, F. Ma, T. Li, H. Yang, J.H.W.M. Rombout, L. An, Molecular characterization of a fish-specific toll-like receptor 22 (TLR22) gene from common carp (*Cyprinus carpio L.*): evolutionary relationship and induced expression upon immune stimulants, Fish Shellfish Immunol. 63 (2017) 74–86.

[33] A.Y. Sundaram, V. Kiron, J. Dopazo, J.M. Fernandes, Diversification of the expanded teleost-specific toll-like receptor family in Atlantic cod, *Gadus morhua*, BMC Evol. Biol. 12 (1) (2012) 256.

[34] A. Rebl, E. Siegl, B. Köllner, U. Fischer, H.-M. Seyfert, Characterization of twin toll-like receptors from rainbow trout (*Oncorhynchus mykiss*): evolutionary relationship and induced expression by Aeromonas salmonicida salmonicida, Dev. Comp. Immunol. 31 (5) (2007) 499–510.

[35] M. Samanta, B. Swain, M. Basu, G. Mahapatra, B.R. Sahoo, M. Paichha, S.S. Lenka, P. Jayasankar, Toll-like receptor 22 in Labeo rohita: molecular cloning, characterization, 3D modeling, and expression analysis following ligands stimulation and bacterial infection, Appl. Biochem. Biotechnol. 174 (1) (2014) 309–327.

[36] 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.

[37] 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.

[38] E. Ringø, 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.

[39] A. Cuesta, M.Á. Esteban, J. Meseguer, *In vitro* effect of chitin particles on the innate cellular immune system of gilthead seabream (*Sparus aurata L.*), Fish Shellfish Immunol. 15 (1) (2003) 1–11.

[40] M.A. Esteban, A. Cuesta, J. Ortuño, J. Meseguer, Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata L.*) innate immune system, Fish Shellfish Immunol. 11 (4) (2001) 303–315.

[41] M.D. Finke, Complete nutrient content of four species of feeder insects, Zoo Biol. 32 (1) (2013) 27–36.

[42] E. Holen, M. Espe, S.M. Andersen, R. Taylor, A. Aksnes, Z. Mengesha, P. Araujo, A co culture approach show that polyamine turnover is affected during inflammation in Atlantic salmon immune and liver cells and that arginine and LPS exerts opposite effects on p38MAPK signaling, Fish Shellfish Immunol. 37 (2) (2014) 286–298.

[43] D.B. Iliev, T. Hansen, S.M. Jørgensen, A. Krasnov, J.B. Jørgensen, CpG- and LPS-activated MAPK signaling in *in vitro* cultured salmon (*Salmo salar*) mononuclear phagocytes, Fish Shellfish Immunol. 35 (4) (2013) 1079–1085.
[44] E. Holen, P. Araujo, N.H. Sissener, G. Rosenlund, R. Waagbø, A comparative study: difference in omega-6/omega-3 balance and saturated fat in diets for Atlantic salmon (*Salmo salar*) affect immune-, fat metabolism-, oxidative and apoptotic-gene expression, and eicosanoid secretion in head kidney leukocytes, Fish Shellfish Immunol. 72 (2018) 57–68.
[45] D. Cao, J. Luo, D. Chen, H. Xu, H. Shi, X. Jing, W. Zang, CD36 regulates lipopolysaccharide-induced signaling pathways and mediates the internalization of Escherichia coli in cooperation with TLR4 in goat mammary gland epithelial cells, Sci. Rep. 6 (2016) 23132 23132.
[46] V. Gómez-Abellán, M.P. Sepulcre, The role of prostaglandins in the regulation of fish immunity, Mol. Immunol. 69 (2016) 139–145.
[47] E. Holen, J. He, M. Espe, L. Chen, P. Araujo, Combining eicosapentaenoic acid, decosahexaenoic acid and arachidonic acid, using a fully crossed design, affect gene expression and eicosanoid secretion in salmon head kidney cells *in vitro*, Fish Shellfish Immunol. 45 (2) (2015) 695–703.
[48] N.S. Liland, I. Biancarosa, P. Araujo, D. Biemans, C.G. Bruckner, R. Waagbø, B.E. Torstensen, E.-J. Lock, Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media, PLoS One 12 (8) (2017) e0183188.
[49] I. Belghit, R. Waagbø, E.-J. Lock, N.S. Liland, Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon, Aquacult. Nutr. 0 (0) (2018).
[50] E. Skřivanová, M. Marounek, G. Dlouhá, J. Kaňka, Susceptibility of Clostridium perfringens to C2–C18 fatty acids, Lett. Appl. Microbiol. 41 (1) (2005) 77–81.
[51] N.A. Dierick, J.A. Decuypere, K. Molly, E. Van Beek, E. Vanderbeke, The combined use of triacylglycerols containing medium-chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition: I. *In vitro* screening of the release of MCFAs from selected fat sources by selected exogenous lipolytic enzymes under simulated pig gastric conditions and their effects on the gut flora of piglets, Livest. Prod. Sci. 75 (2) (2002) 129–142.
[52] T. Spranghers, J. Michiels, J. Vrancx, A. Ovyn, M. Eeckhout, P. De Clercq, S. De Smet, Gut antimicrobial effects and nutritional value of black soldier fly (*Hermetia illucens L.*) prepupae for weaned piglets, Anim. Feed Sci. Technol. 235 (2018) 33–42.
[53] P.A. Olsvik, T. Kristensen, R. Waagbo, B.O. Rosseland, K.E. Tollefsen, G. Baeverfjord, M.H. Berntssen, mRNA expression of antioxidant enzymes (SOD, CAT and GSH-Px) and lipid peroxidative stress in liver of Atlantic salmon (*Salmo salar*) exposed to hyperoxic water during smoltification, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141 (3) (2005) 314–323.
[54] J. Joyner-Matos, D. Abele, J.P.V. Medina, T. Zenteno-Savín, Oxidative stress in aquatic ecosystems: selected papers from the second international conference, Comp. Biochem. Physiol. Mol. Integr. Physiol. 200 (2016) 1–2.
[55] Y. Li, T.M. Kortner, E.M. Chikwati, H.M. Munang'andu, E.-J. Lock, Å. Krogdahl, Gut health and vaccination response in pre-smolt Atlantic salmon (*Salmo salar*) fed black soldier fly (*Hermetia illucens*) larvae meal, Fish Shellfish Immunol. 86 (2019) 1106–1113.
[56] K. Hamre, K. Kolås, K. Sandnes, K. Julshamn, A. Kiessling, Feed intake and absorption of lipid oxidation products in Atlantic salmon (*Salmo salar*) fed diets coated with oxidised fish oil, Fish Physiol. Biochem. 25 (2001) 209–219.
[57] I. Biancarosa, V. Sele, I. Belghit, R. Ørnsrud, E.-J. Lock, H. Amlund, Replacing fish meal with insect meal in the diet of Atlantic salmon (*Salmo salar*) does not impact the amount of contaminants in the feed and it lowers accumulation of arsenic in the fillet, accepted, The Journal of Food Additives and Contaminants, 2019.
[58] L. Bruni, R. Pastorelli, C. Viti, L. Gasco, G. Parisi, Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source, Aquaculture 487 (2018) 56–63.