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Abstract

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Document type : *Article de périodique (Journal article)*

Fish & Shellfish Immunology 47 (2015) 782–796

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Effects of dietary linseed oil on innate immune system of Eurasian perch and disease resistance after exposure to *Aeromonas salmonicida achromogen*

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ARTICLE INFO

Article history:
Received 6 August 2015
Received in revised form
12 October 2015
Accepted 15 October 2015
Available online 20 October 2015

Keywords:
Eurasian perch
Linseed oil
LC-PUFA
Aeromonas salmonicida
Innate immune system
Eicosanoid
Reactive oxygen species
Lysozyme
Alternative complement pathway

ABSTRACT

This study was designated to investigate the effects of dietary fish oil (FO diet) replacement by linseed oil (LO diet) on regulation of immune response and disease resistance in Eurasian perch (*Perca fluviatilis*). A control diet containing fish oil (FO = cod liver oil) and characterized by high levels of n−3 high LC-PUFA (6% EPA, 7.5% of total fatty acids (FAs)) was compared to linseed oil diet (LO diet) composed of low LC-PUFA contents (1% EPA, 2.3% DHA of total FAs) but high C_{18} fatty acids levels. The experiment was conducted in quadruplicate groups of 80 fish each. After 10 weeks of feeding, the innate immune status was evaluated in various organs (liver, spleen, and head-kidney) (feeding condition). Two days later, a bacterial challenge was performed on fish from 2 rearing conditions: fish infected with *Aeromonas salmonicida* (bacteria condition) and fish injected with sterile medium but maintained in the same flow system that fish challenged with bacteria (sentinel condition). Three days after injection of bacteria, a significant decrease of lymphocyte, thrombocyte and basophil populations was observed while neutrophils were not affected. In addition, plasma lysozyme activity and reactive oxygen species production in kidney significantly increased in fish challenged with *A. salmonicida* while the plasma alternative complement pathway activity was not affected. Increase of plasma lysozyme activity as well as reactive oxygen species production in spleen and kidney of sentinel fish suggest that these immune defenses can also be activated, but at lower bacteria concentration than infected fish. No differences in leucocyte populations, plasma lysozyme and alternative complement pathway activities were observed between dietary treatments. Similarly, expression of genes related to eicosanoid synthesis in liver were not affected by the dietary oil source but were strongly stimulated in fish challenged with *A. salmonicida*. These findings demonstrated that the use of linseed oil does not deplete the innate immune system of Eurasian perch juveniles.

1. Introduction

Traditionally, fish oil (FO) is the main lipid source in aquaculture feeds due to its long chain polyunsaturated fatty acids (LC-PUFA) levels including high contents in eicosapentaenoic acid (EPA, 20:5n−3) and docosahexaenoic acid (DHA, 22:6n−3), but also low content in arachidonic acid (ARA, 20:4n−6). A large number of fish species currently farmed are carnivorous and display high dietary requirements in LC-PUFA. Indeed, these species exhibit low endogenous LC-PUFA synthesis capacities as these fatty acids (FAs) are supplied through the ingestion of LC-PUFA-rich prey from their natural environment [1]. However, the use of marine ingredients is not sustainable in the long run since it further reduces the marine fish stocks. In addition, the decrease of marine ingredient availabilities has induced an increase of feed costs with negative consequences on the on-farm production costs. In this economic context, alternative ingredients to replace fish oil in fish feed have been investigated since the 1990's. Until now, vegetable oils (VOs) are considered as the best alternative to marine sources. Nevertheless,

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http://dx.doi.org/10.1016/j.fsi.2015.10.021
1050-4648/

Abbreviations

ACH50	Hemolytic alternative complement
ALA	Alpha-linolenic acid
ALOX5	Arachidonate 5-lipoxygenase
ARA	Arachidonic acid
ATP	Adenosine tri-phosphate
BHI	Brain heart infusion
BHT	Butylated hydroxy toluene
cDNA	Complementary deoxyribonucleic acid
COX	Cyclooxygenase
DHA	Docosahexanoic acid
EF1α	Elongation factor 1 alpha
ELOVL5	Elongation of very long chain 5
EPA	Eicosapentaenoic acid
FA	Fatty acid
FADS	Fatty acid desaturase
FADS2	Fatty acid desaturase 2
FAME	Fatty acid methyl ester
FO	Fish oil
gDNA	Genomic deoxyribonucleic acid
HSI	Hepato-somatic index
IL-1	Interleukin 1
IL-6	Interleukin 6
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acid
LO	Linseed oil
LPS	Lipopolysaccharide
LTB	Leukotriene
LTAH4	Leukotriene a-4 hydrolase
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PLA2	Phospholipase A2
PG	Prostaglandin
PTGES2	Prostaglandin E synthase 2
PUFA	Polyunsaturated fatty acid
RRBC	Rabbit red blood cell
RO	Rapeseed oil
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SD	Standard deviation
SGR	Specific growth rate
SO	Soybean oil
TX	Thromboxane
TNFα	Tumor necrosis factor α
UV	Ultra violet
VAPA	Virulence array protein A
VO	Vegetable oil
WW	Wet weight

contrary to fish oils, vegetable oils are deprived in LC-PUFA but contain a significant proportion of Poly Unsaturated Fatty Acids (PUFA), some being particularly rich in linoleic acid (LNA, 18:2n−6), while a few contain mostly α-linolenic acid (ALA, 18:3n−3). Thus, the use of dietary VOs strongly reduces the LC-PUFAs, as these fatty acids are only supplied by fish meal, but also modify the n−3/n−6 PUFA ratio.

Effects of dietary FA profiles resulting of VO on fish growth performances and its nutritional values (especially on LC-PUFA contents in flesh) have been investigated during the last decades, whereas consequences on fish health and disease resistance are less documented. As demonstrated, dietary FA compositions modulate immune functionality including phagocytosis and macrophage respiratory burst activity, as well as humoral immunological processes such as serum lysozyme and alternative complement activities [2–4]. Indeed, lipids may interfer with immune cell activity through several mechanisms, including changes of physical stability of immune cell membranes (fluidity, membrane-associated enzyme activities and receptor sites) and cell signaling through production of eicosanoids and cytokines. For example, LC-PUFA from phospholipids can be recruited as precursors in eicosanoid production through enzymatic cascades including phospholipase A2 (PLA2), cyclooxygenases (COX1/COX2) and lipooxygenases (FLAP/ALOX5) to produce signaling molecules such as thromboxanes (TX), leukotrienes (LTB) and prostaglandins (PG), themselves involved in inflammation process and other pathway mechanisms of immunity [5]. As demonstrated, EPA is considered as an antagonist to ARA, acting as a competitive substrate for the main enzymes of the eicosanoid pathway. Indeed, in Atlantic salmon (*Salmo salar*), increasing of n−6 PUFA intake has induced an increase of ARA-derived eicosanoids [6] while supplementation of EPA-enriched medium in isolated astrogial cells of turbot (*Scophthalmus maximus*) decreased the production of ARA-derived eicosanoids [7]. In fact, eicosanoids derived from n−6 LC-PUFA have pro-inflammatory and immunoactive functions, whereas eicosanoids derived from n−3 LC-PUFA have anti-inflammatory properties, traditionally attributed to their ability to inhibit the formation of n−6 LC-PUFA-derived eicosanoids [5]. Fish cell membranes usually contain higher amounts of EPA than ARA, but it has been demonstrated that ARA is the preferred FA substrate for the enzymes involved in eicosanoid synthesis [8,9]. In mammals as in fish, ARA is the precursor of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), which are two of the main eicosanoids involved in the regulation of immune functions. LTB4 contributes in the activation of the proliferation of T and B cells, stimulates the release of cytokines from monocytes and T cells such as tumor necrosis factor α (TNF-α) and interleukins (IL1, IL6), acting as a potent chemoattractant and inducing natural killer cell activity [10–12] while PGE_2 is mainly related with pro-inflammatory and immunosuppressive functions. Thus, a well-balanced in n−3/n−6 fatty acid ratio is essential for good fish health and disease resistance.

In fish nutrition, the use of VOs has been shown to induce alterations in several immune parameters, depending on the substitution level, VO source and studied species [13]. In European sea bass (*Dicentrarchus labrax*), dietary VOs significantly decreased the number of circulating leucocytes and the macrophage respiratory burst activity [14], while the use of a full vegetable-based diet significantly increased alternative complement pathway activity and lysozyme activity in plasma [15]. Supplementation of fish oil by rapeseed oil (RO) or soybean oil (SO) in diet decreased the macrophage activity in gilthead seabream (*Sparus aurata*) [4]. More recently, it has also been demonstrated that gilthead seabream fed with VO-based diets exhibited lower basal expressions of interleukine 1β (IL1β) and tumor necrosis factor (TNF-α) cytokines compared with fish fed a FO-based diet [13] and depleted alternative complement pathway activity [16]. In contrast, in Atlantic salmon, the use of linseed oil (LO) did not affect the lysozyme activity, the alternative complement activity and the reactive oxygen species (ROS) production [17]. Similarly, Seierstad et al. [18] did not find

differences in the expression of pro-inflammatory cytokines after stimulation with LPS in Atlantic salmon head kidney leukocytes of fish fed FO, RO or a blend of FO/RO. As previously reported, inclusion of VOs in diets induced strong alterations of several immune parameters in marine fish [3,4,19] while these effects appeared less pronounced in freshwater fish and salmonids [18,20]. However, effects of dietary vegetable oils on fish immunity are often not conclusive or contradictory.

The discrepancy in the modulation of immune parameters among fish species might be related to their own endogenous LC-PUFAs biosynthesis capacities which allow to partially compensating a strong LC-PUFAs deficiency in VO-based diets. Indeed, the LC-PUFA levels in fish tissues are maintained by both dietary input and endogenous LC-PUFAs synthesis. As described in salmonids [21–25] in zebrafish (*Danio rerio*) [26,27], in Nile tilapia (*Oreochromis niloticus*) [27] and in Senegalese sole (*Solea senegalensis*) [28], supplementation of VO in diets induced an activation of LC-PUFAs endogenous biosynthesis while very low endogenous LC-PUFA productions were observed in some marine carnivorous fish such as sea bass [2] and Atlantic cod (*Gadus morhua*) [29]. During the last decades, a dichotomy between freshwater/salmonids and marine fish species has been proposed to explain the differences in LC-PUFA biosynthesis capacities among fish, because of the differences in the abundance of these fatty acids between marine and freshwater environments. However, recent studies suggested that the trophic level of a species could arguably exert a more pronounced influence on the LC-PUFA biosynthesis capacity than the traditional dichotomy "freshwater/salmonids and marine" fish species.

The endogenous LC-PUFA biosynthesis potential was also investigated in the freshwater carnivorous Eurasian perch (*Perca fluviatilis*) because of a recent interest on this species to diversification in the inland aquaculture in Europe [30,31]. The relative high DHA concentrations recorded in flesh of Eurasian perch after a nutritional challenge with low LC-PUFA levels in diets suggested a relatively high endogenous potential of this fish species to synthesize LC-PUFAs from PUFA precursors [31,32]. However, effects of dietary VOs on immune system of Eurasian perch were never investigated.

The present work was part of a wide study on the effects of dietary LO on lipid metabolism and immune functions. The first objective was to characterize the endogenous LC-PUFA synthesis potential of Eurasian perch after a nutritional conditioning of 10 weeks with two experimental diets, a control diet formulated with FO, and a second diet in which FO was replaced by LO [33]. At the end of this nutritional trial, findings from complementary approaches (fatty acid composition, *fads2* and *elovl5* gene expression and FADS2 enzymatic activity) suggest that Eurasian perch can synthesize LC-PUFAs at a significant physiological level. The objective of the present study was to investigate how total replacement of FO by LO in diet for 10 weeks affects basal innate immune system of Eurasian perch and its global immune competence after exposure to Gram-negative bacteria, *Aeromonas salmonicida achromogen*. To this end, leucocyte populations in blood, lysozyme and alternative complement pathway activities in plasma, ROS production in spleen and kidney, and expression of genes related to innate immune system in spleen, kidney and liver were investigated at the end of the nutritional challenge (feeding condition). In a second experiment, these immune parameters were investigated at the end of a bacteria challenge performed under two experimental conditions: fish injected with sterile liquid medium but reared in the same flow system than fish injected with *A. salmonicida* (sentinel condition) and fish injected with bacteria (fish injected with 4.10^7 cfu of bacteria).

2. Materials and methods

2.1. Nutritional trial

Details concerning the nutritional trial are given in Geay et al. [33]. Briefly, Eurasian perch juveniles obtained from the commercial farm Asialor pisciculture (Nancy, France) were randomly distributed into four 100 l fiberglass tanks per dietary treatment at the density of 80 fish per tank. Fish with an initial mean body weight of 17.5 g were reared at 23 °C under a 12L:12D (Light:Dark) photoperiod, and hand fed 3 times per day (except on Sunday) at apparent satiation during 10 weeks.

Two isoenergetic and isonitrogenous diets (18.7 MJ kg^{-1}; 50% crude protein, 11.6% crude lipid) were formulated using two different oil sources (Table 1). The control diet was formulated from cod liver oil (fish oil: FO diet) and characterized by n−3 high LC-PUFA levels, especially EPA (6.0% of total FAs) and DHA (7.5% of total FAs) (Table 2). The second diet, prepared with linseed oil (LO diet), was composed of low LC-PUFA contents (1% EPA and 2.3% DHA of total FAs) but high C_{18} fatty acids levels (48.1% ALA and 14.6% LNA of total FAs) with a n−3/n−6 PUFA ratio close to 3 (Table 2).

During the experiment, mortality was recorded daily. After 10 weeks of feeding, 5 fish were sampled from each replicate tank as described later.

2.2. Bacterial challenge

A strain of *A. salmonicida achromogen* was provided by the Centre d'Economie Rurale (CER) group (Marloie, Belgium). Bacteria

Table 1
Formulation (g kg^{-1}) and chemical composition (% dry matter) in fish oil (FO) and linseed oil (LO) experimental diets.

	FO	LO
Cod fish meal[a]	330	330
Blood meal[b]	80	80
Wheat gluten[c]	80	80
Gelatin[d]	30	30
Starch[d]	200	200
Glucose[d]	25	25
Bacteriologic agar[d]	10	10
Carboxymethylcellulose[d]	50	50
Cellulose[d]	10	10
Cod oil[e]	90	0
Linseed oil[f]	0	90
Vitamin mix[g]	10	10
Mineral mix[h]	65	65
Antioxydant mix[i]	10	10
Betaine[d]	10	10
Proximate composition		
Protein	50.0 ± 0.2	50.3 ± 0.1
Lipid	11.6 ± 0.3	11.6 ± 0.2
Moisture	11.0 ± 0.3	10.3 ± 0.4

BHA, butylated hydroxyanisole; BHT, butylated hydroxyl toluene.
[a] Cod fish meal provided by SNICK euroingredient NV, Ruddervoorde (Belgium).
[b] Actipro Hemoglobin, Zwevezele (Belgium).
[c] Roquette Freres, Lestrem (France).
[d] Sigma–Aldrich, Saint-Louis, MO, (USA).
[e] Mosselman SA, Ghlin (Belgium).
[f] Huilerie Emile Nöel SAS (France).
[g] Vitamin mix was provided by INVE aquaculture. Composition of mixture according to Griffin et al., 1994.
[h] Mineral mix (g $kg^{-1}$of mix) was prepared in the lab, from $(CaHPO_4)2H_2O$, 727.77; $(MgSO_4)7H_2O$, 127.50; NaCl, 60.00; KCl, 50.00; $(FeSO_4)7H_2O$, 25.00; $(ZnSO_4)7H_2O$, 5.50; $(MnSO_4)4H_2O$, 2.54; $(CuSO_4)5H_2O$, 0.78; $(CoSO_4)7H_2O$, 0.48; $(CaIO_3)6H_2O$, 0.29; $(CrCl_3)6H_2O$, 0.13.
[i] 5 g kg^{-1} butylated hydroxyanisole (BHA) and 5 g kg^{-1} butylated hydroxyl toluene (BHT) provided by Fluka, Steinheim (Switzerland).

Table 2
Fatty acid composition (% of total fatty acids) in the FO and the LO experimental diets.

Fatty acids	FO	LO
C14:0	3.2	0.2
C16:0	14.4	7.5
C17:0	0.2	0.1
C18:0	4.0	4.7
C20:0	0.2	0.2
Total saturated	22.0	12.7
C16:1n−7	4.5	0.3
C18:1n−9	26.8	19.0
C18:1n−7	3.1	1.1
C20:1n−9	1.8	0.0
Total monoenes	36.2	20.4
C18:2n−6	17.3	14.6
C18:3n−6	0.2	0.0
C20:3n−6	0.3	0.0
C20:4n−6	0.5	0.2
Total n−6 PUFA	18.4	14.8
C18:3n−3	3.3	48.1
C18:4n−3	0.8	0.0
C20:3n−3	0.2	0.1
C20:4n−3	0.0	0.0
C20:5n−3	6.0	1.0
C22:5n−3	2.4	0.1
C22:6n−3	7.5	2.3
Total n−3 PUFA	20.1	51.7

were cultured in sterile brain heart infusion (BHI) liquid medium (Sigma–Aldrich, Saint-Louis, MO, USA) and incubated at 23 °C in order to follow its growth for 3 days. The relationship between optical density (OD) at 595 nm and bacterial concentration in liquid medium was estimated in several ODs (0.15; 0.33; 0.58; 0.75; 0.92). To this end, 100 μl of culture in each time point was diluted in series in liquid BHI medium and spread in triplicate on solid BHI medium. After 2 days of incubation at 23 °C, clones on each plate were counted to estimate the bacterial concentration corresponding to each OD value.

One day after the end of the nutritional experiment, 160 fish (FO: 47.6 g, LO: 40.9 g) from each experimental diet were randomly selected and transferred in a class 2 confinement facility adapted to conduct bacterial challenges. For each dietary treatment, fish were maintained in eight 100 l glass tanks at the density of 20 fish per tank. In this bacterial challenge, two experimental conditions were investigated: sentinel and bacteria (4 tanks per condition). Fish of sentinel and bacteria groups were reared in the same flow system, but sentinel fish were injected with sterile BHI medium while those of bacteria group were injected with *A. salmonicida achromogen.* Based on the relationship between OD and bacteria concentration, a liquid culture was prepared and stopped when the culture reached an OD of 0.7, corresponding to 4.10^7 cfu/ml. Hundred μl of bacterial solution were injected intraperitoneally to fish anaesthetized with 120 mg l^{-1} MS-222 (aminobenzoic acid). Fish were reared under the same experimental conditions including water temperature (23 °C), photoperiod (12L:12D), feeding method (3 meals/day) and fed the same experimental diets (FO and LO diets). During this experiment, mortality was recorded daily for 3 days. Three days after bacterial injections, 5 fish per tank were randomly collected.

Nutritional trial and bacterial challenge were approved by the local Ethic Committee for Animal Research of the University of Namur (Belgium) (Protocol Number: UN KE 14/213).

2.3. Sampling procedures

In both trials, 5 fish per tank were randomly collected and anesthetized with 120 mg l^{-1} of MS-222. Around 0.5 ml of blood was sampled and mixed with 50 μl heparin (heparin 5000 U.I. ml^{-1}, LEO Pharma B.V., The Netherlands) to avoid blood coagulation. Before centrifugation at 5000 g for 8 min to collect serum for the hemolytic alternative complement (ACH50) and lysozyme assays, an aliquot of 50 μl of total blood was kept on ice to characterize the leucocyte populations. After blood sampling, fish were euthanized with an overdose of MS-222 (240 mg l^{-1}). Around 50 mg of liver, anterior kidney and spleen were sampled from each fish and stored directly at −80 °C for gene expression analysis. The remaining parts of kidney and spleen samples were individually kept on ice for measuring the reactive oxygen species (ROS) production (respiratory burst activity).

2.4. Plasma lysozyme activity

Lysozyme activity was assayed according to the method of Siwicki and Studnica [34] with some modifications. Briefly, 7 μl of plasma were mixed with a freshly prepared *Micrococcus luteus* (Sigma–Aldrich) solution (0.6 mg ml^{-1} Na_2HPO_4 buffer 0.05 M, pH 6.2). Lysozyme assay was performed in triplicate. Absorbance was measured at 450 nm for 30 min. Lysozyme activity (Unit.ml^{-1}) was defined as the amount of enzyme causing an absorbance decrease of 0.001 per min.

2.5. Hemolytic activity of alternative complement pathway (ACH50)

Following the method of Milla et al. [35], ACH50 was evaluated by measuring hemolytic activity of perch serum against rabbit red blood cells (RRBC) (Biomerieux, Marcy-l'Etoile, France). Ten μl of 3% RRBC suspension in veronal buffer (Biomerieux, Marcy-l'Etoile, France) were added to 60 μl of serially-diluted plasma (from 1/66 to 1/1419 in veronal buffer). Total and spontaneous hemolysis was obtained by adding 60 μl of distilled water or veronal buffer to 10 μl RRBC. Plates were incubated at 25 °C for 100 min, mixed every 20 min and finally centrifuged at 2000 g for 10 min. Supernatant was collected and absorbance was measured at 405 nm ACH50 was expressed as the 50% lysis dilution calculated by linear regression.

2.6. Respiratory burst activity of phagocytic cells in spleen and kidney

The procedure used to measure intracellular superoxide production was adapted from Milla et al. [35]. Briefly, the dissected piece of spleen or kidney tissue was weighted and gently mashed with 1 ml of L-15 medium through a 100 μm nylon mesh grid and using the back of a syringe piston. The resulting cell suspension was harvested from the Petri dish, placed in a polypropylene tube and centrifuged at 500 g for 5 min. The supernatant was discarded and 500 μl of fresh L-15 medium was added to each tube and vortexed to resuspend the pellet. This washing operation was performed twice. Then, 100 μl of cell suspension were added to polypropylene tubes in duplicate. Standard curve was made using aliquots of 100 μl from pooled cell suspension. Samples and standard curve were incubated for 30 min and respectively added with 100 μl or increasing volumes (from 0 to 100 μl) of nitroblue tetrazolium (NBT, 2 mg ml^{-1} dissolved in PBS, pH 7.4). Following 1 h-incubation at room temperature, samples and standard curve were centrifuged and supernatants were discarded. Finally, the cells were fixed with 280 ml of methanol and reduced formazan was dissolved in 240 ml of 2 M KOH and 280 ml of N-dimethyl-formamide. After centrifugation at 1200 g, the amount of reduced NBT (i.e. formazan) was determined by spectrophotometry at 550 nm.

2.7. Blood leucocyte populations

Blood leucocyte populations were determined according to the method of Inoue et al. [36] adapted from Ref. [37]. Briefly, a stock solution of 3,3-dihexyloxacarbocyanine (DiOC6(3)) (Sigma–Aldrich, Steinheim, Germany) (500 μg ml^{-1} ethanol) was 10 times diluted in HBSS (Sigma–Aldrich, Steinheim, Germany). Ten μl of heparinized blood were mixed with 1950 μl of HBSS and 40 μl of freshly prepared $DiOC_6$ into a specific FACS analysis tube. After gentle mixing, the tubes were incubated for 10 min at room temperature. Blood cells were analyzed (forward scatter: FSC 1.98 (Lin) side scatter: SSC 528 and green fluorescence: FL-1245) using a FACScalibur apparatus (Becton Dickinson, Erembodegem, Belgium). Red blood cells were separated from white blood cells using a FL-1 vs SSC dot-plot. Then, leucocyte populations were determined using a FSC vs SSC dot-plot and their relative proportions were calculated using Cell Quest Pro™ software. With this technique, distinction was possible between neutrophils, basophils and a mixture of lymphocytes + thrombocytes (monocytes were rare in Eurasian perch blood).

2.8. Gene expression analysis

In each fish, total RNA from kidney, liver and spleen were extracted individually using 1 ml of Extract-all® reagent (Eurobio, Courtaboeuf, France) following manufacturer's instructions and analyzed by electrophoresis in a 1.2% agarose gel to check RNA integrity. After verification of RNA integrity and quantification of nucleic acid concentration with a Nanodrop 2000 spectrophotometer (Thermo Scientific Wilmington, USA), a pool of 20 μg of total RNA corresponding to each tank replicate (n = 4) was constituted from 4 μg total RNA of each individual sample. Each pool of total RNA was treated with RTS DNAse™ kit (MO BIO Laboratories, Carlsbad, CA) to avoid gDNA contaminations. Then, 3 μg of total RNA were reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). At the end of the reverse transcription reaction, cDNA was diluted 20 times in sterile water and kept at −20 °C.

The relative expression of virulence array protein A (*vapA*), leukotriene a-4 hydrolase (*ltah4*), arachidonate 5-lipoxygenase (*alox5*), Prostaglandin E synthase 2 (*ptges2*), complement C3, C-type lysozyme, tumor necrosis factor alpha (*tnf-α*) and the housekeeping genes elongation factor 1 alpha (*ef1-α*) and *βactin* were investigated by real-time quantitative RT-PCR using specific primers (Table 3). Primer sequences were designed with primer3 software and efficiency of each primer set was validated before gene expression analysis (range of reaction efficiencies between 90 and 105%). In this study, *ef1-α* and *βactin* genes were not altered by dietary treatments and conditions (feeding, sentinel and bacteria), and could be used as reference genes. Specific amplification in cDNA samples was performed in triplicate using the iQ™SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycling and fluorescence detection were conducted in a StepOnePlus Real Time PCR System (Applied System) under the following conditions: 10 min of initial denaturation at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. After each run, amplification of single amplicon was confirmed by analyzing the melt curve and sequencing of four PCR products by Macrogen Europe (Amsterdam, The Netherlands). The relative mRNA levels of *VapA*, *tnf-α, C-type lysozyme, complement C3, Alox5, Ptges2 and Ltah4* in each sample were normalized with the geometric mean of *ef1-α and βactin* calculated by the relative standard curve method [38].

2.9. Statistical analysis

The statistical analysis was performed using the Statistica biosoftware (version 8.0). Results are presented as means ± SD. The data were checked for normal distribution and homogeneity of variances by Pearson and Bartlett tests respectively. Percentage values of leucocyte populations were transformed with arc sinus function prior to be analyzed. Effects of dietary treatments and conditions (feeding, sentinel, bacteria) on leucocyte populations, ACH50, lysozyme activity, respiratory burst activity (ROS production) and gene expressions were carried on using a two-way ANOVA. In all statistical analysis test used, $P < 0.05$ was considered statistically different.

3. Results

3.1. Growth performances and mortality

At the end of the 10 week nutritional experiment, fish fed LO diet displayed significantly lower weight gain (134%) than fish fed FO diet (180%) (Table 4), or 1.22 compared to 1.43% day^{-1} in terms of specific growth rate.

Table 3
Primers used for each gene expression analysis by real-time RT-PCR.

Gene	GenBank accession no.	Sense	Primer sequences (5′–3′)	Annealing temperature (°C)	PCR efficiency (%)
VapA	AJ749879	Forward	ATTAGCCCGAACGACAACAC	60.0	102.4
		Reverse	CCAACACAATGAAACCGTTG	58.0	
C-type lysozyme	DR730904	Forward	TGACTGGGTTTGTCTGAGCAAGTG	60.1	97.4
		Reverse	GATGCCATAGTCAGTGGATCCGTC	60.0	
Tnf-α	FJ946993	Forward	AGATCCCCACTACACGTTGAGGCA	61.2	96.1
		Reverse	TTGGAAGCCGCCCTGAGCGAA	63.6	
Complement C3	DR730703	Forward	GTACCAGCTCTTTGGGTGTCAGCA	61.8	93.5
		Reverse	GTAAGCCCTCATGTCCCATAGCAG	62.0	
Ltah4	KR360726	Forward	ACAACCCTCTGACCAACCTG	62.0	94.5
		Reverse	CAGGACGTCCACCTTGTCTT	62.0	
Alox5	KR360727	Forward	TGACAAGGCTAACGCAACAG	60.0	94.4
		Reverse	GTAGCCTCCCACACCCTGTA	64.0	
Ptges2	KR360725	Forward	GGAGATCAAGTGGTCGGTGT	62.0	102.5
		Reverse	CATGCTTCTCTCCGTGTTGA	60.0	
EF1-α	KC513785	Forward	GGAAATTCGTCGTGGATACG	60.0	91.1
		Reverse	GGGTGGTTCAGGATGATGAC	62.0	
βactin	EU664997	Forward	ACCTTCTACAACGAGCTGAGAGTT	50.6	98.2
		Reverse	AGTGGTACGACCAGAGGCATAC	51.6	

Table 4
Growth and survival rates in Eurasian perch juveniles fed FO and LO diets observed at the end of the nutritional trial of 10 weeks and mortality recorded after 3 days of bacterial challenge. Mean values in similar row with different superscript letters are significantly different (Student *t*-test, $P < 0.05$).

		FO diet		LO diet	
Nutritional conditioning	Initial weight (g)	17.5 ± 0.2		17.4 ± 0.2	
	Final weight (g)	47.6 ± 1.2^{b}		40.9 ± 4.5^{a}	
	Weight gain (%)	180 ± 8^{b}		134 ± 22^{a}	
	Mortality (%)	4.7 ± 3.0		5.0 ± 1.0	
Bacterial challenge		Sentinel	Bacteria	Sentinel	Bacteria
	Mortality (%)	0	35	0	37

In the bacterial challenge, no mortality was observed after 3 days in fish injected with sterile BHI medium (sentinel condition) for both dietary treatments (Table 4). In contrast, injection of *A. salmonicida* 4.10^{7} cfu induced a cumulative mortality of 35% and 37% after 3 days in FO diet and LO diet respectively.

3.2. Blood leucocyte populations

The leucocyte population in blood significantly decreased in fish of sentinel and bacteria groups in comparison with feeding group (Fig. 1). However, in the sentinel group, only fish fed FO diet exhibited a significantly lower leucocyte population compared with fish fed FO in this group. Among leucocyte cells, lymphocytes + thrombocytes and basophiles populations significantly decreased in fish of sentinel and bacteria groups compared with feeding group, while neutrophil/eosinophil populations were not affected (Fig. 1). Globally, the leucocyte populations were not modulated by the dietary treatments (Table 5).

3.3. Plasma ACH50 and lysozymes activities

In plasma, the activity of alternative complement pathway was not significantly modulated between dietary treatments and conditions (feeding, sentinel and bacteria) (Fig. 2). Similarly, the lysozyme activity measured in plasma was not significantly affected by oil source whereas its activity significantly increased in sentinel and bacteria groups, compared with feeding group (Fig. 2, Table 5).

3.4. Reactive oxygen species production (respiratory burst activity) in spleen and kidney

ROS production significantly increased in spleen and kidney of fish of sentinel groups. Injection of bacteria also induced an increase of ROS production in kidney but not in spleen. In this assay, the ROS production was not significantly modulated by the dietary treatments (Table 5).

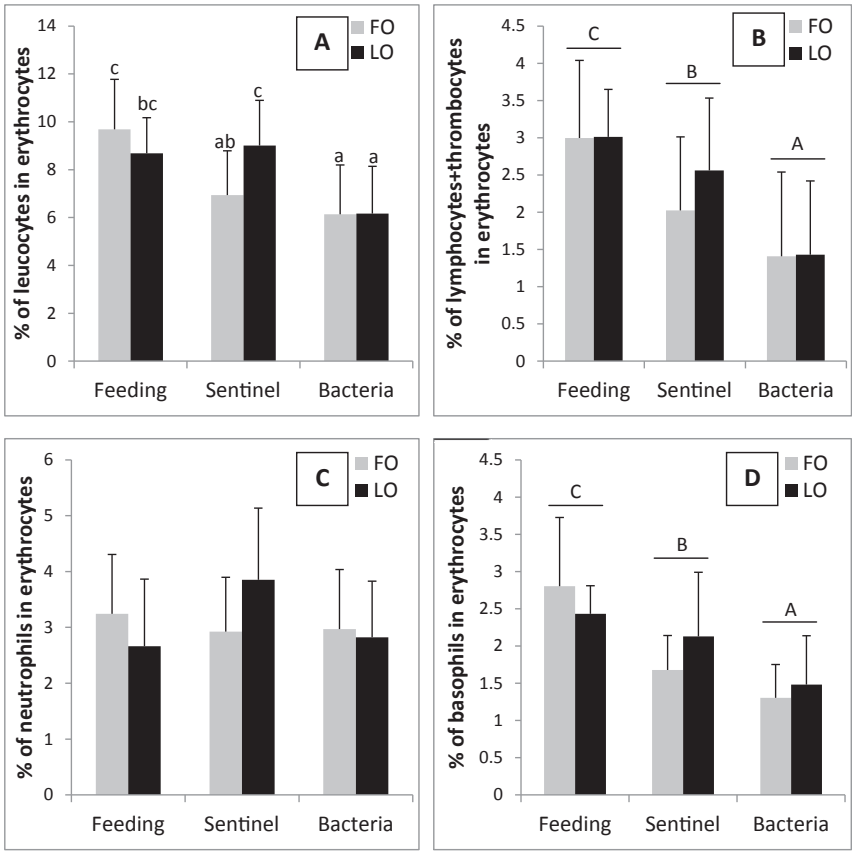

Fig. 1. Blood leucocyte (A) lymphocyte + thrombocyte (B), neutrophil (C) and basophil (D) populations expressed in percentages of total blood cells from fish fed FO and LO diets and after 3 days of injections of challenged with *Aeromonas salmonicida achromogens* compared to feeding or sentinel fish. Values are expressed as mean ± SD of twenty fish. Statistical differences ($P < 0.05$) between dietary treatments are indicated by different Greek letters, statistical differences ($P < 0.05$) between conditions (feeding, sentinel and bacteria) are indicated by different capital letters, while significant interactions (diets × conditions, $P < 0.05$) are indicated by lower capitals.

Table 5
Results of the two-way analysis of variance.

		Factors		
	Variables	Diet	Condition	Diet × condition
Leucocyte populations	Leucocytes	NS	*	*
	Lymphocytes + thromocytes	NS	*	NS
	Neutrophils	NS	NS	NS
	Basophils	NS	*	NS
Enzymatic activities	Lysozyme activity	NS	*	NS
	ACH50	NS	NS	NS
	ROS spleen	NS	*	NS
	ROS kidney	NS	*	NS
Gene expression in kidney	VapA	NS	*	NS
	Tnf-α	NS	NS	NS
	C-type lysozyme	NS	*	*
	Complement C3	NS	NS	NS
	Alox5	NS	*	NS
	Ptges2	NS	NS	*
	Ltah4	NS	NS	*
Gene expression in spleen	VapA	NS	*	NS
	Tnf-α	NS	*	NS
	C-type lysozyme	NS	NS	NS
	Complement C3	NS	NS	*
	Alox5	NS	NS	NS
	Ptges2	NS	NS	NS
	Ltah4	NS	NS	NS
Gene expression in liver	VapA	NS	*	NS
	Tnf-α	*	NS	NS
	C-type lysozyme	*	*	NS
	Complement C3	*	*	NS
	Alox5	NS	*	NS
	Ptges2	NS	*	NS
	Ltah4	NS	*	NS

Significance levels are * $P < 0.05$ and NS $P > 0.05$.

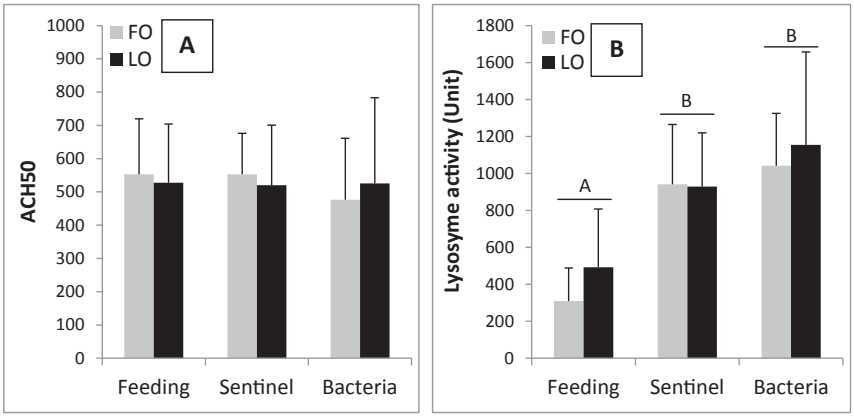

Fig. 2. Hemolytic activity of alternative complement pathway (ACH50) (A) and lysozyme activity (B) measured in plasma from fish fed FO and LO diets and reared under feeding, sentinel and bacteria conditions. Values are expressed as mean ± SD of twenty replicates. Statistical differences ($P < 0.05$) between dietary treatments are indicated by different Greek letters, statistical differences ($P < 0.05$) between conditions (feeding, sentinel and bacteria) are indicated by different capital letters, while significant interactions (diets × conditions, $P < 0.05$) are indicated by lower capitals.

3.5. VapA, tnf-α, C-type lysozyme, complement C3, alox5, ptges2 and Ltah4 relative gene expression in kidney, spleen and liver

The relative expression of *vapA* involved in the virulence of *A. salmonicida* was investigated in kidney, spleen and liver (Figs. 4–6) in order to detect the presence of this pathogen in the tissues. No *vapA* transcripts were detected in these tissues from fish of feeding group. In sentinel group, very low expression levels of *vapA* gene were measured in liver, spleen and kidney. Fish injected with *A. salmonicida* displayed high *vapA* expression levels in liver, kidney and spleen, without significant differences between dietary treatments.

Among genes of immune system investigated, *tnfα* involved in inflammatory response was not modulated by diet and group factors in kidney (Fig. 4). In spleen, *tnfα* expression was significantly down regulated in fish belonging to the bacteria group, compared with fish feeding and sentinel groups whereas its expression was not affected by dietary treatments (Fig. 5). On the contrary, *tnfα* expression in liver was significantly up regulated in fish fed LO diet whereas its expression was not significantly different between groups (feeding, sentinel and bacteria) (Fig. 6).

C-type lysosyme expression was not modulated by diet and group factors in spleen (Fig. 5) while it was stimulated in kidney of fish fed FO diet and injected with bacteria (Fig. 4). In liver, *C-type*

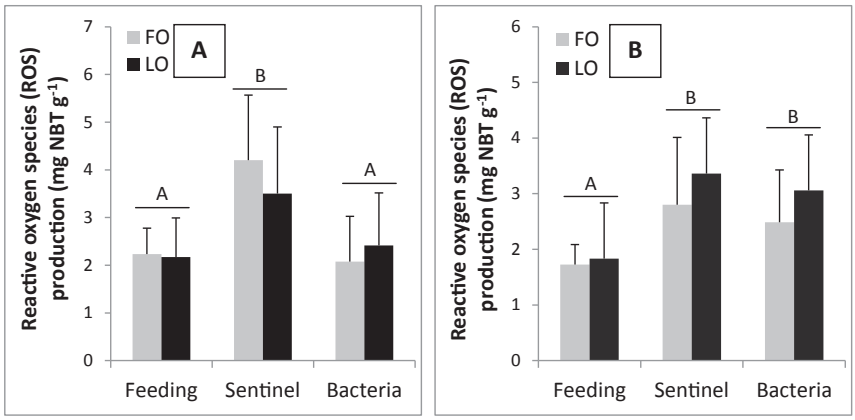

Fig. 3. Reactive oxygen species (ROS) production in spleen (A) and kidney (B) from fish fed FO and LO diets and reared under feeding, sentinel and bacteria conditions. Values are expressed as mean ± SD of twenty replicates. Statistical differences ($P < 0.05$) between dietary treatments are indicated by different Greek letters, statistical differences ($P < 0.05$) between conditions (feeding, sentinel and bacteria) are indicated by different capital letters, while significant interactions (diets × conditions, $P < 0.05$) are indicated by lower capitals.

lysosyme expression was down regulated in the bacteria group in comparison with feeding and sentinel groups (Fig. 6). In addition, *C-type lysosyme* expression was globally stimulated in fish fed LO diet, irrespective of the group conditions.

The expression of *complement C3* was not modulated by diet and group factors in kidney (Fig. 5) whereas its expression was globally stimulated in liver of fish fed LO diet, irrespective of the group conditions (Fig. 6). *Complement C3* expression in liver was stimulated in fish injected with bacteria in comparison with feeding and sentinel groups.

Among genes involved in eicosanoid synthesis, *alox5* expression in kidney and liver was stimulated in fish of bacteria group, in comparison with fish feeding and sentinel groups (Figs. 4 and 5). Similarly, *ptges2* and *ltah4* expression levels in liver were significantly higher in fish injected with bacteria in comparison with feeding and sentinel conditions (Fig. 6). In addition, *ltah4* expression in kidney of bacteria group was significantly higher in fish fed FO diet than in fish fed LO diet (Fig. 4).

Standardization of RT-qPCR conditions and the lack of significant differences in the absolute gene expression of both housekeeping genes (*ef1-α* and *βactin*) between tissues allowed comparing the relative expression of interest genes between organs. Genes of the immune system displayed contrasted expression patterns between the tested organs. *Complement C3* and *C-type lysozyme* genes were strongly expressed in liver in comparison with kidney and spleen (Figs. 4–6). On the contrary, *alox5* and *tnf-α* genes were mainly expressed in spleen and kidney whereas high levels of *lath4* transcripts were detected in liver and kidney. Finally, the highest relative *ptges2* expression level was observed in liver.

4. Discussion

In the present study, Eurasian perch juveniles were fed for 10 weeks with two experimental diets which differed in their oil source, fish oil rich in LC-PUFAs including high contents in EPA and DHA, or linseed oil containing low LC-PUFA contents (1% EPA, 2.3% DHA of total FAs) but high C_{18} fatty acids levels. At the end of the nutritional trial, fish fed LO diet displayed a significantly lower weight gain (134%) than fish fed FO diet (180%), while the mortality was low in all tanks and not affected by oil source. In addition, dietary LO significantly decreased the EPA, ARA and DHA concentrations and modified the n−3/n−6 LC-PUFA balance in liver and muscle phospholipids at the end of the nutritional experiment (shown in Geay et al. [33]). To evaluate the impacts of dietary FO replacement by LO on the regulation of Eurasian perch immune defense, fish were challenged with *A. salmonicida achromogen*, a Gram-negative bacteria previously isolated in Eurasian perch farming where it was responsible of strong mortality (Dr. F. Lieffrig, CER Marloie, personal com.).

Three days after bacterial injection, no mortality was observed in sentinel fish while in fish infected with bacteria, 35% and 37% of mortality rates were recorded in fish fed FO diet and LO diet respectively. Similarly, in Nile tilapia, no differences in cumulative mortalities were observed 3 days and 15 days between fish fed FO or LO diets, after injection with *Streptococcus iniae* [39]. On the contrary, bacterial challenge with *A. salmonicida* spp. *salmonicida* in Artic charr (*Salvelinus alpinus*) revealed a higher mortality (48%) in fish fed diet supplemented with FO in comparison with fish fed LO diet (37%) or soybean (SO) diet (20%) after 30 days of challenge [40]. Similarly, Fracalossi and Lovell [41] and Li et al. [42] found that high n−3 PUFA concentrations in diets also reduced survival rate of channel catfish (*Ictalurus punctatus*) after a challenge with *Edwardsiella ictaluri*. In Atlantic salmon, Bransden et al. [43] found significantly increased cumulative mortalities when fish fed diets containing sunflower oil and challenged with *Vibrio anguillarum* in comparison with fish fed FO after 22 days of bacterial challenge. These findings may indicate a large variability in the key role of a well n−3/n−6 PUFA dietary balance to improve immune system and disease resistance in fish. Indeed, carnivorous fish species, including Eurasian perch, displayed high n−3/n−6 PUFA dietary ratio requirement which can be maintained by the use of LO rich in ALA (C18:3n−3), while this high n−3/n−6 PUFA ratio is less adapted to other fish species including channel catfish in which relatively high dietary n−6 PUFA are required. Thus, in the present study, the absence of differences in the cumulative mortalities between dietary treatments after 3 days of challenge might reflect an appropriate n−3/n−6 PUFA balance supply by LO diet. However, as observed in some of the experiments previously cited, differences of cumulative mortalities between dietary treatments were only observed after 5–30 days of bacterial challenge, according to the quantity of bacteria injected, the pathogen virulence, and the rearing conditions [39,40].

In this experiment, the use of LO in replacement to FO did not modulate the total leucocyte population as well as each blood leucocyte population at the end of the nutritional trial (Fig. 1). In contrast, a significant reduction in the percentage of total leucocytes in blood was previously reported in European sea bass fed with diets in which 60% of dietary FO was replaced by LO, reflecting

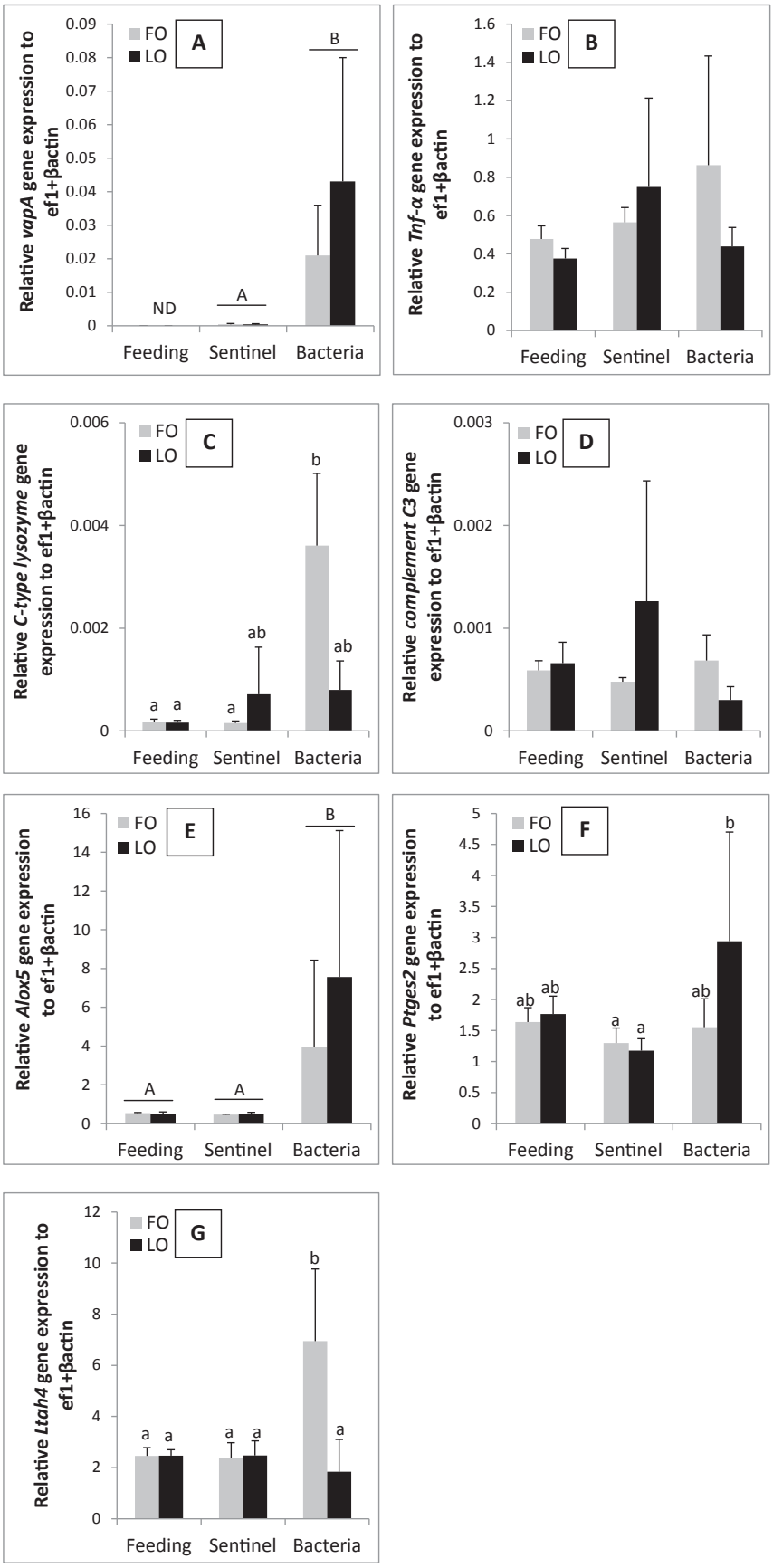

Fig. 4. *VapA* (A), *tnf-α* (B), *C-type lysozyme* (C), *complement C3* (D), *Alox5* (E), *Ptges2* (F) and *Ltah4* (G) relative gene expression measured in kidney from fish fed FO and LO diets and reared under feeding, sentinel and bacteria conditions. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef1-α* and *βactin* gene expressions. *VapA* transcripts were not detected (ND) in feeding condition. Values are expressed as mean ± SD of four replicates, each originating from a blend of five tissues. Statistical differences ($P < 0.05$) between dietary treatments are indicated by different Greek letters, statistical differences ($P < 0.05$) between conditions (feeding, sentinel and bacteria) are indicated by different capital letters, while significant interactions (diets × conditions, $P < 0.05$) are indicated by lower capitals.

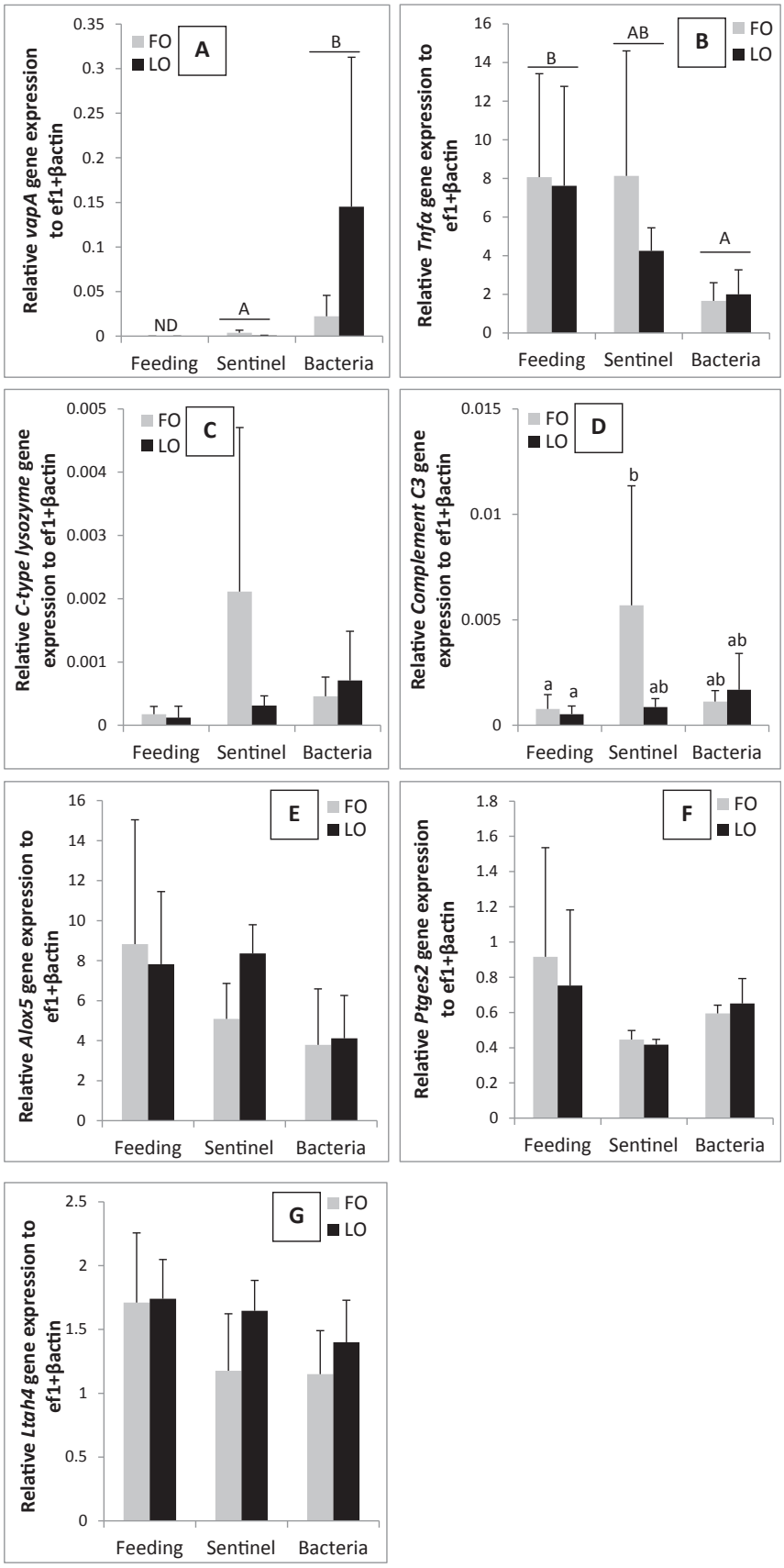

Fig. 5. *VapA* (A), *tnf-α* (B), *C-type lysozyme* (C), *complement C3* (D), *Alox5* (E), *Ptges2* (F) and *Ltah4* (G) relative gene expression measured in spleen from fish fed FO and LO diets and reared under feeding, sentinel and bacteria conditions. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef1-α* and *βactin* gene expressions. *VapA* transcripts were not detected (ND) in feeding condition. Values are expressed as mean ± SD of four replicates, each originating from a blend of five tissues. Statistical differences ($P < 0.05$) between dietary treatments are indicated by different Greek letters, statistical differences ($P < 0.05$) between conditions (feeding, sentinel and bacteria) are indicated by different capital letters, while significant interactions (diets × conditions, $P < 0.05$) are indicated by lower capitals.

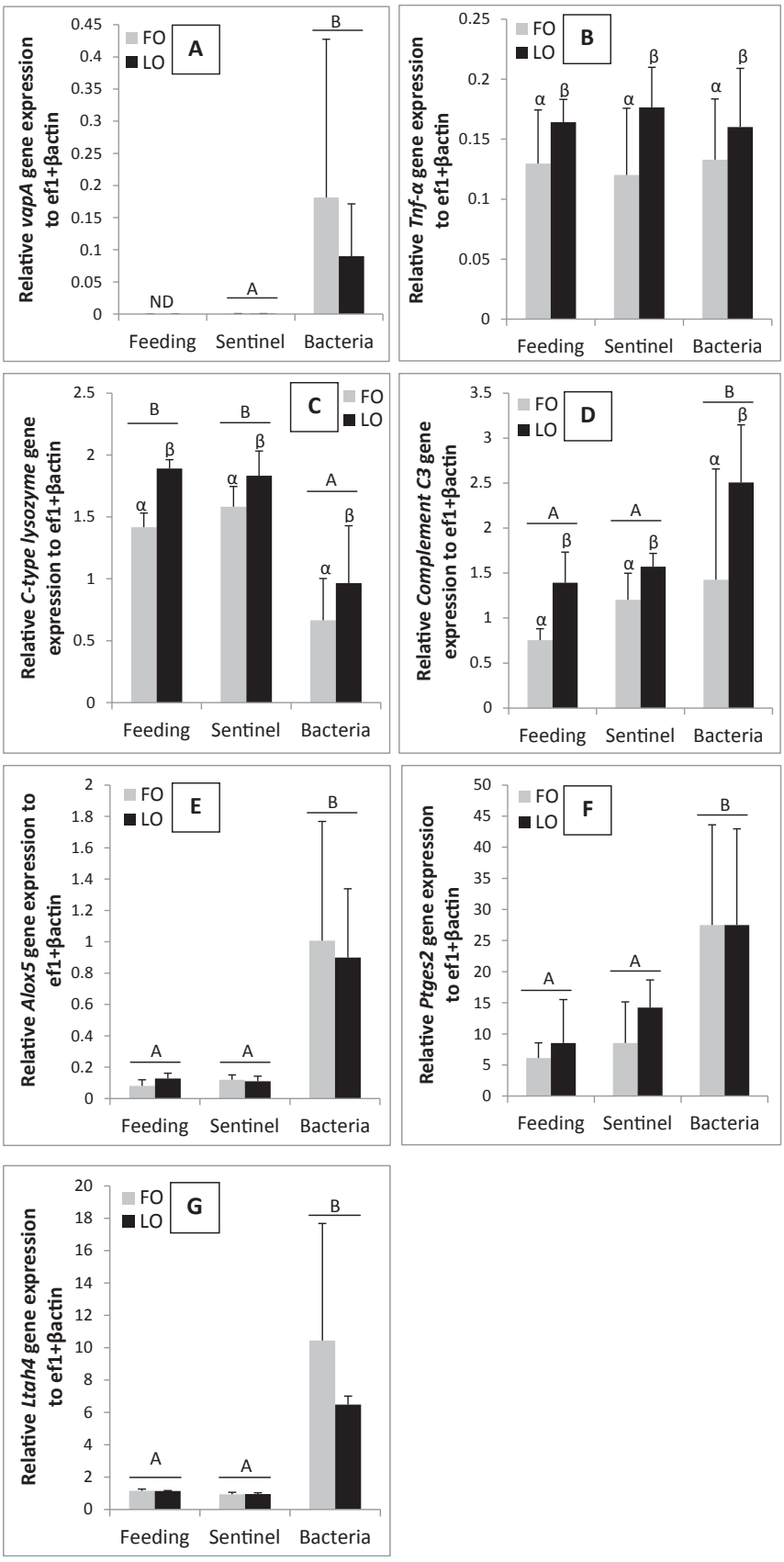

Fig. 6. *VapA* (A), *tnf-α* (B), *C-type lysozyme* (C), *complement C3* (D), *Alox5* (E), *Ptges2* (F) and *Ltah4* (G) relative gene expression measured in liver from fish fed FO and LO diets and reared under feeding, sentinel and bacteria conditions. Relative transcript (mRNA) levels were determined by real-time RT-PCR and normalized by the arithmetic mean of *ef1-α* and *βactin* gene expressions. *VapA* transcripts were not detected (ND) in feeding condition. Values are expressed as mean ± SD of four replicates, each originating from a blend of five tissues. Statistical differences ($P < 0.05$) between dietary treatments are indicated by different Greek letters, statistical differences ($P < 0.05$) between conditions (feeding, sentinel and bacteria) are indicated by different capital letters, while significant interactions (diets × conditions, $P < 0.05$) are indicated by lower capitals.

a putative immunodeficiency in these fish [14,19]. In the present bacterial challenge, a significant decrease of the total leucocyte concentrations in blood was observed in fish challenged with *A. salmonicida* but also in fish of sentinel condition. As observed in Fig. 1, this decrease of total blood leucocytes may be explained by a decrease of the lymphocytes, thrombocytes and basophil populations. This observation could indicate a depletion of these leucocyte populations in response to *A. salmonicida*. In contrast, neutrophil population was not affected in fish injected with bacteria and sentinel groups, suggesting a key role of this leucocyte population in disease resistance.

The consequences of dietary LO on the humoral innate immune system of Eurasian perch were investigated through the measure of ACH50 and lysozyme activities in plasma as well as the relative *complement c3* and *lysozyme C-type* gene expressions in kidney, spleen and liver. The complement has several functions but it is most well-known for its capacity to destroy pathogens by creating pores in their surface membranes and to opsonize pathogens for destruction by phagocytes [44]. Moreover, in mammals, complement can modulate adaptive immune system after binding on lymphocyte membranes. Alternative complement pathway, one of the three complement pathways, is antibody independent and can be directly activated by the lipopolysaccharide (LPS) present on the surface of Gram-negative bacteria such as *A. salmonicida*, and thus, inducing its lysis [45].

As indicated in Fig. 2, no difference of basal ACH50 activity levels in plasma was observed between dietary treatments at the end of the nutritional trial. This result is also supported by the lack of difference in the relative *complement c3* gene expression between diets in liver, kidney and spleen in fish of the feeding group (Figs. 4–6). However, globally, a higher *complement c3* expression level was observed in liver of fish fed LO diet (Table 5), which might be related to an imbalance in liver PUFA contents in these fish, as reported by Geay et al. [33]. Similarly, the basal serum ACH50 activity was not affected by the total replacement of FO by rapeseed oil (RO) in black carp (*Mylopharyngodon piceus*) [46] and by LO or safflower oil in rainbow trout [47]. By contrast to these freshwater species, total substitution of FO by LO or soybean oil in diet decreased significantly the serum ACH50 activity in gilthead seabream [48], while its activity was enhanced in European sea bass fed with a blend of VOs [15]. The discrepancies between fish species may reflect an imbalance of LC-PUFA in some of them when dietary FO was replaced by VO. However, the differences in the modulation of ACH50 activity between fish species may also be related to genetic variation between teleosts [49]. Indeed, contrary to mammals in which a single C3 gene was identified, variable number of *complement C3* isoforms encoded by paralogue genes was found in fish species. For example, catfish, rainbow trout and seabream possessed respectively one, four and five complement C3 protein isoforms, encoded by different genes [49]. Thus, differences in the number of *c3* genes between fish teleosts might differentially modulate the ACH50 activity among species.

Surprisingly, ACH50 activity was not modulated when fish were challenged with *A. salmonicida*. On the other hand, in gilthead seabream challenged with *Photobacterium damselae*, an increase of the bactericidal activity was observed after 3 days in fish fed FO and LO diets [13]. The lack of ACH50 stimulation in our study may be explained by the strain of bacteria used in this bacterial challenge. Indeed, as explained by Ourth and Bachinski [50], pathogens with high amount of sialic acid such as *A. salmonicida*, are less able to activate complement than non-pathogenic Gram-negative bacteria which have lower sialic acid content.

As observed with ACH50 activity, the basal level of lysozyme activity in plasma was not affected by dietary oil source at the end of the nutritional challenge (feeding condition). Similar result was obtained in pikeperch (*Sander lucioperca*), another Percidae, when dietary FO was replaced by VOs (including LO) [51]. In other freshwater species including rainbow trout [47] and black carp [46], the use of full VO diets did not modulate the basal lysozyme activity in comparison with their respective control diet. On the other hand, while no effects were observed in European sea bass [19] when 60% of dietary FO was replaced by VO (including LO), the use of a full VO-based diet significantly decreased the basal lysozyme activity in this species [15].

A significant increase of plasma lysozyme activity was recorded in fish challenged with *A. salmonicida* (bacteria condition) but also in the sentinel group. This finding suggests that sentinel fish were also contaminated passively through release of bacteria in water. A passive contamination through water was previously observed in Artic charr reared with “cohabitant” fish contaminated with 6.10^3 cfu of *A. salmonicida* [40]. Indeed, 50% mortality was recorded after 8 days in fish exposed to bacteria but mortality was also observed in “sentinel” fish after 18 days, reflecting an indirect contamination. In the present study, Eurasian perch were exposed to a higher bacterial concentration (4.10^7 cfu) and displayed faster and higher mortality after 3 days. Consequently, a relatively high amount of bacteria might be released in water, and thus, contaminated fish of sentinel groups. This hypothesis is supported by the detection of low *vapA* transcript levels in liver, kidney and spleen of sentinel fish. Indeed, *vapA* gene encodes a protein involved in the virulence of *A. salmonicida*. In the present experiment, contamination of fish with *A. salmonicida achromogen* was investigated from primers specifically designed to amplify *vapA* gene of *A. salmonicida achromogen* strain, based on Rattanachaikunsopon and phumkhachorn [52] results. Similar amplitude levels of plasma lysozyme activity between sentinel and bacteria groups suggest that low concentration of *A. salmonicida* is able to activate this innate immune parameter. Enhanced of plasma lysozyme activity was also observed in carp (*Cyprinus carpio*) challenged with *Aeromonas punctata* [34] or with the protozoan *Eimeria subepithelialis* [53], and in Atlantic salmon infected with *A. salmonicida* [54]. However, in the present study, increase of plasma lysozyme activity was not associated to a stimulation of the *c-type lysozyme* expression in liver, kidney and spleen. Moreover, the significantly higher *c-type lysozyme* expression level observed in liver of fish fed LO diet (Table 5) was not associated to a higher plasma lysozyme activity in these fish. These observations may be explained by the fact that plasma lysozyme is not only synthesize by *c-type lysozyme* gene, but also depends of the goose-type lysozyme (*g-type lysozyme*) gene, which was not investigated in the present experiment.

As observed in Fig. 2, the dietary oil source did not affect the amplitude of plasma lysozyme activity in bacteria groups. Similarly, FO replacement by corn oil or LO in the diet did not result in a difference of serum lysozyme activity in Nile tilapia when fish were infected with *S. iniae* [39]. On the contrary, gilthead seabream fed with a LO-based diet exhibited a significantly higher serum lysozyme activity after infection with *P. damselae* than fish fed control diet (FO) [13]. However, as reported by Saurabh and Sahoo [55], a negative genetic correlation was demonstrated between disease resistance and serum lysozyme activity level in several fish species including rainbow trout, Atlantic salmon and Nile tilapia. Indeed, in these experiments, higher mortalities after pathogen challenges were observed in fish which displayed the highest serum lysozyme activity, reflecting a detrimental effect of high lysozyme levels on disease resistance of fish. Thus, in the present study, the absence of differences in the plasma lysozyme activity levels between fish fed FO diet and LO diet may suggest that the use of LO did not deplete disease resistance of Eurasian perch.

In addition to plasma ACH50 and lysozyme humoral immune parameters, the reactive oxygen species (ROS) production, a

bactericidal agent, was investigated in kidney and spleen (Fig. 3). ROS production was not significantly different between dietary treatments at the end of the nutritional trial (feeding group). This finding is supported by previous experiments in which total FO replacement by VO sources did not affect the basal ROS production in head kidney of Atlantic salmon [18] and rainbow trout [47]. Similarly, Thompson et al. [56] found no differences in phagocytosis and bactericidal activities of head kidney macrophages in Atlantic salmon fed diets enriched with either n−3 or n−6 PUFA. In contrast, partial substitution of FO by VO (60%) decreased strongly the basal ROS production in head kidney of European sea bass [14,19] and gilthead seabream [4]. As reported by Wu and Chen [57] in juvenile grouper (*Epinephelus malabaricus*) and Montero et al. [58] in gilthead seabream, imbalance in n−3/n−6 affected ROS production.

In the present experiment, ROS production level significantly increased in fish of sentinel and bacteria groups, in the same amplitude between dietary treatments. This increase of ROS levels in kidney and spleen as well as the increase of plasma lysozyme activity in sentinel groups, in which low *A. salmonicida* were detected, confirmed that these immune parameters are crucial actors in disease resistance which can be rapidly activated as soon as pathogens are detected, even at low concentrations. Moreover, absence of differences in the ROS production levels between dietary treatments may suggest that LO did not induce an immunodeficiency in these fish. As reported by Montero et al. [58] in gilthead seabream and by Waagboo et al. [59] in Atlantic cod, a selective incorporation of certain FAs such as DHA and ARA was observed in head kidney macrophages in order to maintain an appropriate n−3/n−6 balance. Indeed, FA in macrophage membrane cell are essential components as they promote membrane flexibility, enhance phagocytic ability, and others immune processes. Nevertheless, fatty acid compositions in spleen and kidney were not analyzed in the present study.

As reported in rainbow trout, seabream, turbot, goldfish and catfish, TNF-α is a pro-inflammatory cytokine which causes activation of macrophages, leading to increased respiratory burst [60–62]. In the present experiment, *tnf-α* gene was mainly expressed in spleen in comparison with kidney and liver. Thus, the global significant higher *tnf-α* gene expression level recorded in liver of fish fed LO may have low consequences on the global TNF-α synthesis in fish. However, it is interesting to point out that *tnf-α* gene as well as *c-type lysozyme* and *complement c3* genes previously mentioned, were only modulated in liver in which significant modification of PUFA contents were observed between dietary treatments. However, *tnf-α* gene was not simulated in fish infected with *A. salmonicida* (sentinel and bacteria groups) despite the increase of ROS production in these tissues. In contrast, increase of TNF-α after pathogen infections was previously reported in fish [63,64]. In gilthead seabream challenged with *P. damselae*, *tnf-α* gene in head kidney was stimulated one day after infection whereas its expression deceased at the third day [13]. Perhaps, pro-inflammatory cytokine synthesis such as TNF-α can be reaching over expression in lymphoid tissues only few hours after infection, and then, decrease after 3 days. This hypothesize may explain the lack of *tnf-α* gene expression stimulation in infected during the present bacterial challenge.

As previously reported, changes in dietary fatty acid composition have been shown to affect eicosanoid synthesis [14,65]. Eicosanoids are mainly produced from C_{20} LC-PUFA (EPA and ARA) of leucocyte phospholipids. They are catalyzed by the action of cyclooxygenase and lipooxygenase (ALOX5), resulting in signaling molecules that include prostaglandins (PG), leukotriene (LT) and lipoxins that are known to influence a wide range of immune processes [11]. However, the regulation of genes related to eicosanoids in response to pathogen infections is poorly documented [66]. In the present study, the regulation of *alox5*, *ptges2* and *ltah4* expressions in response to dietary oil source and bacterial infection was investigated. As indicated in Figs. 4–6, *alox5*, *ptges2* and *ltah4* genes displayed contrasted basal expression pattern between tissues (feeding group). Indeed, *alox5* and *ltah4* genes were mainly expressed in kidney and spleen while high *ptges2* transcripts were detected in liver, and then in kidney. The strong expression of *alox5* and *ltah4* genes in lymphoid tissues underlines the key role of leucocytes in eicosanoid synthesis. In the feeding groups, FO replacement by LO did not affect the expression *alox5*, *ptges2* and l*tah4* genes, suggesting a well n−3/n−6 PUFA balance in these tissues. Indeed, as mentioned above, selective retention of certain FAs by leucocytes may occur and thus, allowing to maintain an appropriate balance in phospholipid FAs [65]. The regulation of eicosanoid related genes in response to FA dietary contents, especially balance in n−3/n−6 PUFA, is poorly documented, while modulation of PGE_2 and LTB_4 levels in plasma by FA dietary contents have already been investigated in fish species. Ganga and coworkers [67] demonstrated in gilthead seabream that dietary FO substitution by LO did not affect plasma PGE_2 level but significantly decrease plasma PGE_3 level, a prostaglandin derived from EPA. In European sea bass, partial replacement of FO by LO (60%) did not affect plasma PGE_2 level [14] whereas, on the contrary, the same authors identified a significant decrease of PGE2 in this species with the same dietary substitution [19]. In contrast, total replacement of FO by soybean oil did not affect both LTB_4 and PGE_2 levels in head kidney of Atlantic salmon [68].

Interestingly, contrasted regulation of *alox5*, *ptges2* and *ltah4* genes were observed between tissues in response to bacterial infection. In liver, *alox5*, *ptges2* and *ltah4* genes were strongly stimulated in fish of bacteria groups, whereas a significant decrease of their expression was observed in spleen of bacteria group as well as in sentinel group. In addition, no differences between dietary treatments were observed in these tissues. In kidney, *alox5* and *ptges2* genes were significantly stimulated in both dietary treatments of bacteria groups, while increase of *ltah4* transcript level was only observed in fish of bacteria group fed with FO diet. These findings showed the relative different sensitivity of each tissues toward bacterial infection. In addition, the stimulation of *alox5*, *ptges2* and *ltah4* genes in kidney and liver in fish infected with *A. salmonicida* demonstrated the key role of eicosanoids in the innate immune response. The lack of *ltah4* gene stimulation in kidney of fish fed LO diet in bacteria groups might reflect an imbalance in EPA/ARA in this organ. However, the lack of information on the fatty acid composition in kidney does not allow confirming this hypothesis.

In summary, the results from the present study showed that total replacement of dietary FO by LO lowered growth rate but did not affect the immune status as evidenced by various humoral immune variables and some key-gene transcripts. In addition, infection of Eurasian perch with *A. salmonicida* revealed that lysozyme activity, ROS production and genes related to eicosanoid synthesis were modulated in the same amplitude between dietary treatments, reflecting an analogue capacity of fish to fight against pathogens. These findings suggest that the use of LO in Eurasian perch farming could be applied without impairing fish immune functions and disease resistance. However, longer dietary trials associated with bacterial challenges would be necessary to confirm the lack of immunodeficiency in Eurasian perch fed with VOs.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

This work was supported by the Belgian National Funds for Scientific Research (FNRS-FRFC 6807370), including a postdoctoral fellowship to F. Geay and two PhD fellowships to E. Tinti and J. Mellery, and by the IAP project Aquastress (contract P7-31). We thank Kévin Debes of Asialor for supplying Eurasian perch juveniles.

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