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Characterization of the impact of dietary immunostimulant CpG on the expression of mRNA biomarkers involved in the immune responses in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Infectious diseases have significantly impacted Atlantic salmon aquaculture worldwide. Modulating fish immunity with immunostimulant-containing functional feeds could be an effective strategy in mitigating disease problems. Previously, we characterized the impact of polyriboinosinic polyribocytidylic acid (pIC) and formalin-killed typical *Aeromonas salmonicida* bacterin on miRNA expression in Atlantic salmon fed a commercial diet with and without immunostimulant CpG. A set of miRNA biomarkers of Atlantic salmon head kidney responding to pIC and/or bacterin immune stimulations was identified (Xue et al., 2019) [1]. Herein, we report a complementary qPCR study that investigated the impact of the pIC, bacterin and dietary CpG on the expression of immune-relevant mRNAs (n = 31) using the same samples as in the previous study (Xue et al., 2019) [1]. Twenty-six of these genes were predicted target transcripts of the pIC- and/or bacterin-responsive miRNAs identified in the earlier study. The current data showed that pIC and/or bacterin stimulations significantly modulated the majority of the qPCR-analyzed genes involved in various immune pathways. Some genes responded to both stimulations (e.g. *tnfa*, *il10rb*, *ifng*, *irf9*, *cxcr3*, *campb*) while others appeared to be stimulation specific [e.g. *irf3*, *irf7a*, *il1r1*, *mxa*, *mapk3* (pIC only); *clra* (bacterin only)]. *A. salmonicida* bacterin stimulation produced a strong inflammatory response (e.g. higher expression of *il1b*, *il8a* and *tnfa*), while salmon stimulated with pIC showed robust interferon responses (both type I and II). Furthermore, the current data indicated significant down-regulation of immune-relevant transcripts (e.g. *tlr9*, *irf5*, *il1r1*, *hsp90ab1*, *itgb2*) by dietary immunostimulant CpG, especially among pre-injection and PBS-injected fish. Together with our prior miRNA study, the present research provided complementary information on Atlantic salmon anti-viral and anti-bacterial immune responses and on how dietary CpG may modulate these responses.

1. Introduction

Infectious diseases have resulted in substantial mortality and losses to Atlantic salmon (*Salmo salar*) aquaculture worldwide, affecting the growth and sustainability of the industry [2]. Several well-known viruses that cause diseases in Atlantic salmon include salmonid alphavirus (SAV), infectious salmon anemia virus (ISAV), viral hemorrhagic septicemia virus (VHSV), piscine orthoreovirus (PRV), piscine myocarditis virus (PCMV), and infectious pancreatic necrosis virus (IPNV) [3–5]. Bacterial pathogens that have a severe impact on salmonid

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aquaculture include Gram negative bacteria such as *Piscirickettsia salmonis* [6], *Aeromonas salmonicida* [7], *Moritella viscosa* [8], and Gram positive bacteria such as *Renibacterium salmoninarum* [9]. For instance, in Chile, the National Fisheries and Aquaculture Service estimated that about 50 % of disease-causing mortalities in Atlantic salmon were attributed to piscirickettsiosis (a disease caused by *P. salmonis*) in 2020 [10]. The annual direct and indirect losses in the Chilean aquaculture industry due to piscirickettsiosis were approximately USD 700 million [11,12].

One important strategy to fight disease problems in intensive culture systems is to manipulate immune responses of cultured fish using immunostimulant-containing functional feeds [13,14]. Immunostimulants may include intact microbes (e.g. probiotic organisms) and/or microbial cell components (e.g. lipopolysaccharide). These substances generally have repeat structures, often referred to as pattern associated molecular patterns (PAMPs) and can be recognized by pattern recognition receptors (PRRs) within host immune cells, triggering downstream immune responses [15]. Unmethylated DNA containing cytosine-phosphate-guanine oligodeoxynucleotides motifs (CpG ODNs), often found in bacterial genomes as well as in some viral and invertebrate genomes, are essential immunomodulators that can induce or enhance host Toll-like receptor 9 (TLR9)-mediated immune responses [16,17]. Synthetic CpG ODNs are divided into three classes (i.e., A-, B- and C-classes) with distinct immunomodulating properties based on their backbone structure and sequences [18]. For example, CpG ODN 1668 (i.e., B-class), was shown to activate immune responses against iridovirus infection in rock bream (*Oplegnathus fasciatus*) [19], and *Vibrio parahaemolyticus* infection in Pacific red snapper (*Lutjanus peru*) [20]. In addition, previous studies showed protection against sea lice (*Lepeophtheirus salmonis*) infection in Atlantic salmon by orally administered CpG ODN 1668 [21,22].

miRNAs are a group of small (usually 20–24 nucleotides), non-coding RNAs that inhibit gene expression at the post-transcriptional level by binding to partially complementary sequences in their target mRNAs [23–25]. A growing body of literature indicates that miRNAs play critical roles in diverse biological processes such as development, tissue differentiation and regeneration, growth, reproduction and responses to environmental stimuli [23]. In addition, the potential regulatory role of miRNAs in fish immune responses has been reported in a number of species by identifying the miRNAs that are differentially expressed between control and challenged animals [1,26–31]. For example, our previous work [1] showed that the analyses of head kidneys from Atlantic salmon injected with immunogens to elicit antiviral or antibacterial responses [polyriboinosinic polyribocytidylic acid (pIC, a PAMP-like synthetic dsRNA analogue or viral mimic) or formalin-killed typical *Aeromonas salmonicida* (a PAMP-containing bacterin)] revealed 12 and 18 miRNAs differentially expressed in pIC and bacterin groups, respectively, compared to the PBS controls. Further, the functional annotations of predicted target genes of the pIC- and/or bacterin-responsive miRNAs revealed 130 immune-relevant target genes [1]. The pathways mapped to these target genes included NOD-like receptor signaling pathway, cytokine–cytokine receptor interaction, necroptosis, Toll-like receptor signaling pathway, apoptosis, C-type lectin receptor signaling pathway, RIG-I-like receptor signaling pathway, and cell adhesion molecules (CAMs). The impact of dietary CpG ODN 1668 on the expression of miRNAs associated with antiviral and antibacterial responses in Atlantic salmon was also evaluated; the expression levels of several miRNAs (e.g., miR-146a-1-2-3p, miR-192a-5p, miR-194a-5p) in head kidney were significantly affected by dietary CpG [1].

In the present study, we investigated the expression response of 31 selected immune-relevant mRNA biomarkers in the same materials as were used in the aforementioned miRNA study [1]. They were all key immune system genes with functions that, in general, allowed them to be divided into three groups. Twelve were among genes encoding PRRs or involved in PRR signaling pathways, eight were genes encoding cytokines or involved in cytokine mediated pathways, while the remaining eleven genes encoded immune effectors and regulators. These biomarkers were applied to study the effect on the immune system associated with differences in the feed when comparing fish fed diets with and without immunostimulant CpG. Their immune responses to pIC and bacterin while fed the different diets were also investigated aiming to explore the effect of functional feed when host immune responses were triggered by these viral and bacterial mimics [32,33]. The majority of the selected mRNA biomarkers have been predicted as target genes of the pIC and bacterin-responsive miRNAs [1]. The expression profiles of these miRNAs and the predicted targets among the biomarkers were therefore explored to elucidate their possible interactions when responding to same conditions (feed, pIC and bacterin). Finally, the head kidney was selected as the target tissue because it plays a crucial role in both specific and non-specific defense mechanisms in teleost fish. Its role in hematopoiesis is analogous to that of bone marrow in higher vertebrates [34–36].

2. Materials and methods

2.1. Feed production, feeding trial, immune challenge and fish sampling

The production of the feeds used in the current study was described previously [1]. Briefly, EWOS Dynamic S feed (5 mm; 27 % fat, 46 % protein) was used in this experiment as the Control diet and base feed for the functional diet (referred to as CpG diet). The CpG diet was top-coated with 10 mg kg^{-1} of CpG ODN 1668 (Integrated DNA Technologies, Coralville, IA, USA).

The detailed information and procedures related to the feeding trial, immune challenge and fish sampling were described in the previous investigation [1]. Briefly, PIT (passive integrated transponder)-tagged Atlantic salmon smolts (232 ± 52 g mean initial weight ± SD; n = 67) were allocated to four 620 L tanks (16–17 fish per tank) connected to a flow-through seawater system (~10–11 °C, dissolved oxygen ≥10 mg L^{-1}) under a 24 h light photoperiod in the Dr. Joe Brown Aquatic Research Building [JBARB, Ocean Sciences Centre (OSC), Memorial University of Newfoundland, St. John's, NL, Canada]. After acclimation, each dietary treatment was randomly assigned to 2 replicate tanks. Fish were fed to apparent satiation using automatic feeders for seven weeks.

At the end of the feeding trial, both dietary groups were subjected to immune challenge by an intraperitoneal (IP) injection of *A. salmonicida* bacterin, viral mimic pIC or phosphate-buffered saline (PBS). Four fish per tank were euthanized with an overdose of MS-222 (400 mg L^{-1}, Syndel Laboratories, Vancouver, BC, Canada) and dissected for pre-injection head kidney samples. Formalin-killed typical *A. salmonicida* bacterin was obtained in the form of a vaccine (Furogen Dip, Elanco (formerly Novartis), Charlottetown, PE, Canada). The *A. salmonicida* bacterin suspension in sterile PBS was prepared as in Hori et al. [37], while the pIC (Catalogue #P0913; Sigma-Aldrich, Oakville, ON, Canada) was diluted in sterile PBS (Gibco, Carlsbad, CA, USA) at 2 μg μL^{-1} for injection. Then, 4–5 fish from each tank (i.e. 8-9 fish per injection) were anesthetized (50 mg L^{-1} of MS-222) and injected with 1 μL of pIC, bacterin or PBS per g of body weight. Fish were then sampled 24 h post-injection as described above, and head kidney samples (50–100 mg) were collected, flash-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. This study was carried out in accordance with animal care protocol 17-77-MR, approved by the Institutional Animal Care Committee of Memorial University of Newfoundland.

2.2. RNA isolation

Total RNAs of all collected head kidney samples were previously extracted using the *mirVana* miRNA isolation kit (Ambion/Life Technologies, Carlsbad, CA, USA) according to manufacturer instructions [1]. Aliquots of *mirVana*-prepared total RNAs (40 μg) were further treated with DNase I (Qiagen) to degrade residual genomic DNA and

then purified using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocols. The RNA integrity was verified by 1 % agarose gel electrophoresis, and RNA purity was assessed by A260/280 and A260/230 using NanoDrop spectrophotometry (Thermo Fisher, Mississauga, ON, Canada). All RNA samples used in this study showed high integrity (i.e., tight 18S and 28S ribosomal RNA bands) and purity (i.e., A260/230 ratios >1.8 and A260/280 ratios >2.0).

2.3. Gene selection

A total of 31 genes were selected to study the effect of the different feeds on immune pathways at the constitutive level or after activation by immunogens (i.e. pIC or bacterin). The genes were selected based on their known functions and could be grouped into three categories: genes encoding pattern recognition receptors (PRRs) or involved in PRR signaling pathways (n = 12), genes encoding cytokines or involved in cytokine mediated pathways (n = 8), genes playing key roles as immune effectors or regulators (n = 11). A complete overview of all selected biomarkers is given in Table 1.

In our previous investigation [1], small RNA deep sequencing was performed to identify pIC- and/or *A. salmonicida* bacterin-responsive miRNAs in the same materials as investigated in the present study. The study identified 12 and 18 miRNAs differentially expressed in pIC and bacterin groups compared with the PBS controls, respectively. The putative target genes of the pIC- and/or bacterin-responsive miRNAs were predicted, applying the 3′ UTRs from all Atlantic salmon transcripts available in the NCBI Reference Sequence database (Refseq; https://www.ncbi.nlm.nih.gov/refseq/) in 2019 as input in the target predictions. Twenty-three of the 31 selected biomarkers were predicted as targets for one or more of the pIC/bacterin-responsive miRNAs from this study. Following the full-length sequencing of the Atlantic salmon transcriptome (GIYK01000000) [38], and the generation of the Atlantic salmon miRNA target gene database (MicroSalmon, http://github.com/AndreassenLab/MicroSalmon/) [39], the eight remaining biomarkers could be analyzed against the pIC/bacterin-responsive miRNAs. This revealed that three more biomarkers could be added as predicted target genes (*tlr9, myd88, mxb)*. A complete overview of which of the pIC/bacterin-responsive miRNAs that are predicted to regulate 26 of the chosen biomarkers is given in Table 1.

2.4. qPCR analysis

The qPCR experiment included a total of 67 head kidney RNA samples from pre-injection, and PBS-, bacterin-, and pIC-injected fish fed the control or CpG diet (8–9 samples per treatment). The qPCR experiment was performed as previously described [40]. Briefly, First-strand cDNA templates for qPCR were synthesized from 1 μg of DNaseI-treated, column-purified total RNA using random primers (250 ng; Invitrogen/Life Technologies), dNTPs (0.5 mM final concentration; Invitrogen/Life Technologies) and M-MLV reverse transcriptase (200 U; Invitrogen/Life Technologies) with the manufacturer's first strand buffer (1 × final concentration) and DTT (10 mM final concentration) at 37 °C for 50 min. PCR amplifications were performed in 13 μL reactions using 1 × Power SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies), 50 nM of both the forward and reverse primers, and the indicated cDNA quantity (see below). The real-time qPCR analysis program for all primer pairs consisted of 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The sequences of all primer pairs used in qPCR analyses are presented in Supplemental Table S1. Each primer pair was quality-tested using the ViiA 7 Real Time PCR system (Applied Biosystems/Life Technologies). Amplification efficiencies [41] were calculated using three cDNA template pools prepared post-cDNA synthesis: one pool of 4 PBS-injected samples, one pool of 4 pIC-injected samples, and one pool of 4 bacterin-injected samples. The reported efficiencies (Supplemental Table S2) are an average of the three cDNA pools. Standard curves were generated using 5-point 1:3 dilution series starting with cDNA representing ten ng of input total RNA.

Six candidate normalizer genes [i.e. 60S ribosomal protein 32 (*rpl32*; BT043656), β-actin (*actb*; BG933897), elongation factor 1-alpha 2 (*ef1a2*; BT058669), eukaryotic translation initiation factor 3 subunit D (*eif3d*; GE777139), polyadenylate-binding protein 1 (*pabpc1*; EG908498) and ATP-binding cassette sub-family F member 2 (*abcf2*; BT071904)] were tested and analyzed using *geNorm*. *ef1a2* (geNorm M = 0.198) and *pabpc1* (geNorm M = 0.196) were selected as the normalizer genes in this study.

qPCR analyses of the target genes were performed using the ViiA 7 Real Time PCR system (Applied Biosystems/Life Technologies) with diluted cDNAs corresponding to 5 ng of input RNA. The GOIs and normalizer genes were tested in triplicate and a no-template control for each target was included. The relative quantity (RQ) of each transcript was determined using a qBase relative quantification framework [40,42]. The RQs of each GOI were first calibrated to the sample that had the lowest normalized gene expression. For pre-injection samples, the RQs of each GOI were re-calibrated against fish fed the Control diet, while for IP-injected groups, the RQs of each GOI were re-calibrated against PBS-injected fish fed the Control diet.

2.5. Statistical analyses

All qPCR data (i.e., RQs) were subjected to Grubbs' test to identify potential outliers and then $\log_2$-transformation to meet the normality assumption. The normality of the qPCR data (i.e., $\log_2$ RQ values) was analyzed using the Shapiro-Wilk and Kolmogorov-Smirnov normality tests. In total, 36 RQ values were identified as statistical outliers in the entire dataset (i.e., out of 2077 RQ values), and excluded from the study. Each mRNA of interest had a minimum of 7 samples per diet × injection treatment group. For pre-injection samples, differences in mRNA expression between diet groups were evaluated using Student's *t*-test ($p < 0.05$). For IP-injected groups, miRNA expression differences between injection treatments and diets were analyzed using two-way analysis of variance (ANOVA), followed by a Dunnett's test to examine the effect of immunogens within each dietary group (i.e., pIC/bacterin *vs* PBS), and a Student's *t*-test to assess the dietary effect within injection treatment groups ($p < 0.05$). All statistical tests above were conducted using Prism v7.0 (GraphPad Software Inc., La Jolla, CA, USA). Principal coordinates analysis (PCoA), permutational multivariate ANOVA (PERMANOVA), and similarity of percentages analysis (SIMPER) were performed using PRIMER (Version 6.1.15; PRIMER-E Ltd, Ivybridge, UK) to explore diet- and injection treatment-related gene expression changes considering the entire qPCR dataset. All variables were standardized by the total of each variable in PRIMER prior to these multivariate analyses.

3. Results

3.1. Effects of pIC and A. salmonicida bacterin treatments on the expression of immune-relevant genes

As expected, the comparisons between the PBS- and pIC/bacterin-injected salmon revealed multiple changes in the expression of the targeted antiviral and antibacterial biomarker genes. Figs. 1–3 summarize the results from the analysis of the three biomarker categories. The qPCR results of 12 transcripts encoding PRRs or involved in PRR signaling pathways are shown in Fig. 1. Eight of these transcripts (*tlr5a*, *tlr7*, *tlr9*, *myd88*, *irf3*, *irf5*, *irf7a*, *irf7b*) had significant induction by pIC injection when compared with the PBS-injected salmon fed the respective diet (Fig. 1A–C, E-I). The expression of *irf7b* in response to pIC was more strongly induced (up to 54-fold) than the other seven genes including another *irf7* paralogue (i.e. *irf7a*) (up to 15-fold). Five transcripts encoding PRRs [*tlr5a*, *clra*, *irf5* (fish fed CpG diet only), *irf7b* and *tbk1* (fish fed Control diet only)] were significantly induced by bacterin injection compared with the PBS-injected salmon fed the respective diet

Table 1
31 key genes selected as biomarkers by their function in the Atlantic salmon immune system.

Immune-relevant genes	GenBank accession number	Functional annotation[a]	pIC-responsive miRNA[b]	Bacterin-responsive miRNA[b]
Transcripts encoding pattern recognition receptors (PRRs) or involved in PRR signaling pathways				
toll-like receptor 5a (*tlr5a*)[c]	AY628755, GIYK01039966.1	Toll-like receptor signaling pathway; defense response to bacterium	N/A	N/A
toll-like receptor 7 (*tlr7*)[c]	HF970585, GIYK01056395.1	Toll-like receptor signaling pathway; defense response to virus	N/A	N/A
toll-like receptor 9 (*tlr9*)[d]	NM_001123653, GIYK01031897.1	Toll-like receptor signaling pathway; unmethylated CpG binding	miR-462-3p miR-462a-3	miR-183-2-3p
C type lectin receptor A (*clra*)[e]	NM_001123579, GIYK01005171.1	PRR recognizing carbohydrate patterns present on the surface of microorganisms (Zhang et al., 2000)	miR-135bd-5p	N/A
MYD88 innate immune signal transduction adaptor (*myd88*)[d]	NM_001136545, GIYK01061007.1	MyD88-dependent Toll-like receptor signaling pathway	N/A	miR-725-3p
interferon regulatory factor 3 (*irf3*)[e]	BT059292, GIYK01047197.1	type I interferon signaling pathway	miR-8159-5p	miR-725-5p
interferon regulatory factor 5 (*irf5*)[e]	NM_001139852, GIYK01043337.1	positive regulation of type I interferon production	miR-135bd-5p miR-462a-3p miR-462b-3p	N/A
interferon regulatory factor 7a (*irf7a*)[e,f]	NM_001136548	type I interferon signaling pathway	N/A	miR-192a-5p
interferon regulatory factor 7b (*irf7b*)[e,f]	FJ517644, GIYK01025625.1	type I interferon signaling pathway	N/A	N/A
TANK-binding kinase 1 (*tbk1*)[e]	NM_001256722	regulation of type I interferon production	miR-146a-5p	miR-146a-5p
suppressor of IKK-epsilon (*sike1*)[e]	NM_001140308, GIYK01039381.1	inhibitory role in virus-triggered TLR3-dependent interferon activation pathways (Huang et al., 2005)	miR-146a-1-2-3p miR-8159-5p	miR-146a-1-2-3p miR-146a-3-3p miR-183-2-3p
mitogen-activated protein kinase kinase kinase 8 (*map3k8*)[e]	NM_001173785, GIYK01060074.1	transducing signals from TLRs to regulate TNF and IL1B production (Mielke et al., 2009).	N/A	miR-192a-5p miR-200b-3p miR-725-3p miR-725-3p
Transcripts encoding cytokines or involved in cytokine mediated pathways				
interleukin 1 beta (*il1b*)[c]	AY617117, GIYK01053969.1	inflammatory response; cytokine-mediated signaling pathway	N/A	N/A
tumor necrosis factor alpha (*tnfa*)[e]	NM_001123617	inflammatory response; tumor necrosis factor-mediated signaling pathway	miR-8159-5p	miR-146d-1-3p miR-192a-5p miR-725-3p
interleukin 8a (*il8a*; alias *cxcl8a*)[e]	NM_001140710	inflammatory response; induction of positive chemotaxis	N/A	miR-194a-5p
interleukin-1 receptor type I (*il1r1*)[e]	NM_001123633, GIYK01056034.1	inflammatory response; interleukin-1-mediated signaling pathway	N/A	miR-725-5p miR-novel-16-5p
interleukin-10 receptor beta chain precursor (*il10rb*)[e]	BT059022, GIYK01050348.1	inflammatory response; interleukin-10 receptor activity	miR-221-5p	miR-183-1-3-3p miR-183-2-3p miR-221-5p miR-29b-2-5p miR-429ab-3p
interferon gamma (*ifng*)[e]	AJ841811	receptor signaling pathway via JAK-STAT; defense response to virus	N/A	miR-725-3p miR-727a-3p
interferon regulatory factor 9 (*irf9*)[e]	NM_001173719, GIYK01047330.1	regulating the downstream expression of ISGs within the type I IFN response pathway (Paul et al., 2018)	miR-135bd-5p miR-30e-1-2-3p	N/A
C-X-C chemokine receptor type 3 (*cxcr3*)[e]	NM_001140493, GIYK01014915.1	chemokine-mediated signaling pathway; regulation of leukocyte migration	miR-8159-5p	N/A
Transcripts playing key roles as immune effectors or regulators				
interferon-induced GTP-binding protein Mx a (*mxa*)[e]	NM_001123690, GIYK01030883.1	antiviral innate immune response; response to type I interferon	miR-181a-5-3p	N/A
interferon-induced GTP-binding protein Mx b (*mxb*)[d]	NM_001139918, GIYK01030881.1	antiviral innate immune response; response to type I interferon	miR-8159-5p	N/A
interferon stimulated gene 15a (*isg15a*)[e]	BT049918	defense response to virus; response to type I interferon	N/A	N/A
cathelicidin antimicrobial peptide b (*campb*)[e]	NM_001123573	antibacterial humoral response; cellular response to interleukin-1	N/A	miR-183-1-3-3p
B-cell lymphoma 6 protein homolog (*bcl6*)[e]	NM_001140313, GIYK01041444.1	positive regulation of apoptotic process; regulation of immune system process	miR-27d-1-5p	miR-194a-5p
MAP kinase-activated protein kinase 3 (*mapk3*)[e]	NM_001139792, GIYK01051614.1	MAPK cascade; apoptotic process	N/A	miR-146d-1-3p miR-183-2-3p miR-29b-2-5p miR-725-3p miR-novel-16-5p
tumor necrosis factor receptor superfamily member 6 precursor (*tnfrsf6*; alias *fas*)[e]	NM_001173649	extrinsic apoptotic signaling pathway	miR-135bd-5p	miR-192a-5p miR-722-3p
BCL2/adenovirus E1B interacting protein 3-like (*bnip3l*)[e]	NM_001141679, GIYK01028019.1	positive regulation of apoptotic process	miR-146a-5p	miR-146a-5p miR-29b-2-5p
dynamin-1-like protein (*dnm1l*)[e]	NM_001173563, GIYK01040194.1	positive regulation of apoptotic process	miR-462a-3p miR-462b-3p	N/A
heat shock protein HSP 90-beta (*hsp90ab1*)[e]	NM_001146473, GIYK01016018.1	chaperone-mediated protein complex assembly; MHC class II protein complex binding	N/A	miR-200b-3p
integrin beta-2 (*itgb2*)[e]	NM_001165324, GIYK01055469.1	cell adhesion mediated by integrin; neutrophil migration	miR-146a-5p	miR-146a-5p miR-183-2-3p miR-29b-2-5p

[a] Genes were functionally annotated based on selected gene ontology (GO) terms from *Homo sapiens* putative orthologues or published studies.

[b] pIC- or bacterin-responsive miRNAs predicted to target a given candidate gene.
[c] Genes noted with N/A (i.e. not applicable) were not predicted target mRNAs of pIC- and/or bacterin-responsive miRNAs.
[d] Additional target predictions (n = 3) of the pIC- and/or bacterin-responsive miRNAs were carried out in MicroSalmon.
[e] pIC- and/or bacterin-responsive miRNAs were identified and their predicted targets (n = 23) were conducted in Xue et al. [1].
[f] *irf7* paralogues are located on different chromosomes (ssa16 for *irf7a* and ssa10 for *irf7b*), and they are 89.4 % identical at the nucleotide level.

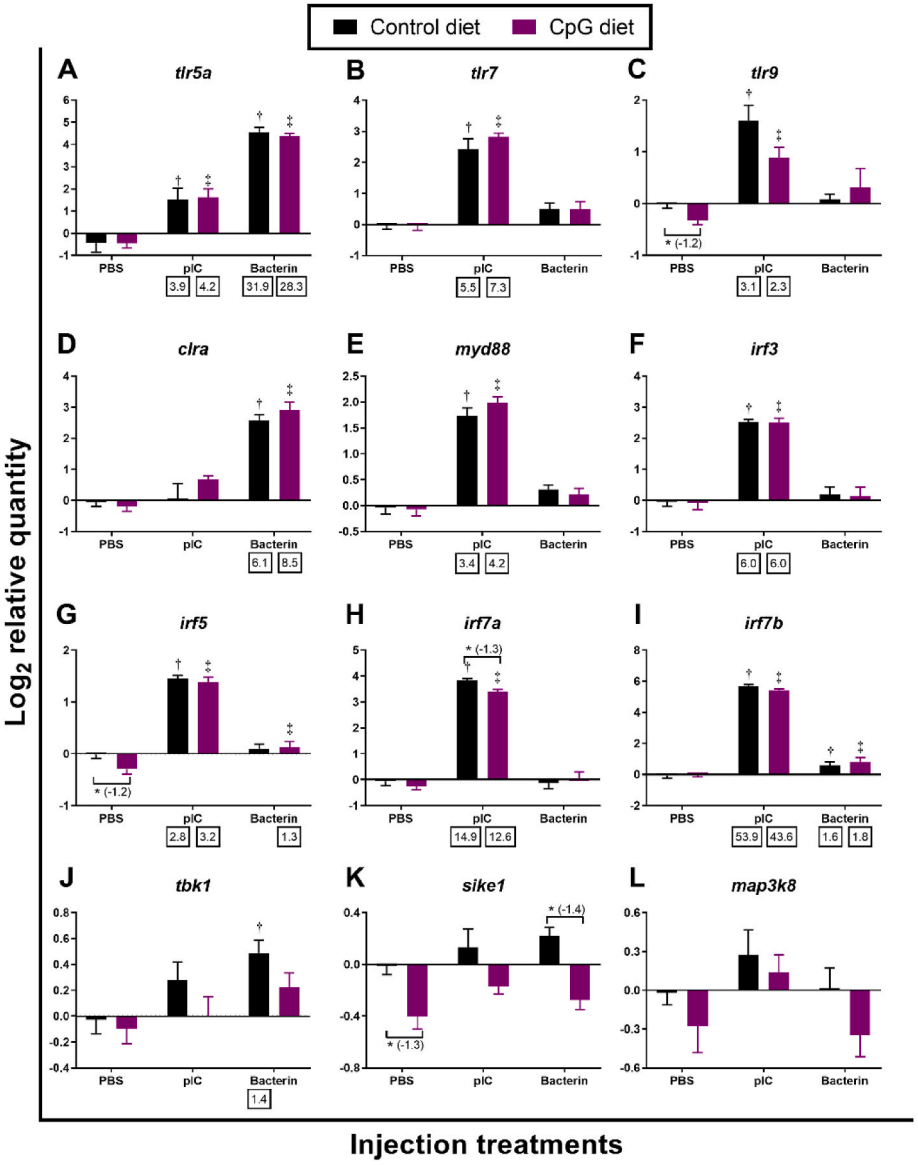

Fig. 1. qPCR analyses of transcripts encoding pattern recognition receptors (PRRs) or involved in PRR signaling pathways (n = 8–9). Average $\log_2$ RQs with SE bars are plotted. An asterisk (*) represents a significant difference between diets in each injection treatment ($p < 0.05$) with fold-change given in brackets. Fold-change between diets was calculated as 2^{A-B}, where A is the mean of $\log_2$ RQ from the CpG fed group, and B is the mean of $\log_2$ RQ from the injection-matched control fed group. For down-regulated mRNAs, fold-change values were inverted (−1/fold-change). A dagger (†) or diesis (‡) represents a significant difference between the pIC/*A. salmonicida* bacterin-injected salmon and the diet-matched PBS-injected control ($p < 0.05$) with fold-change indicated below the x-axis. Fold up-regulation or down-regulation between injections was calculated as 2^{A-B}, where A is the mean of $\log_2$ RQ from the pIC or bacterin groups, and B is the mean of $\log_2$ RQ from the diet-matched PBS group. (**A**) *toll-like receptor 5a*; (**B**) *toll-like receptor 7*; (**C**) *toll-like receptor 9*; (**D**) *C type lectin receptor A*; (**E**) *MYD88 innate immune signal transduction adaptor*; (**F**) *interferon regulatory factor 3*; (**G**) *interferon regulatory factor 5*; (**H**) *interferon regulatory factor 7a*; (**I**) *interferon regulatory factor 7b*; (**J**) *TANK-binding kinase 1*; (**K**) *suppressor of IKK-epsilon*; (**L**) *mitogen-activated protein kinase kinase kinase 8*.

(Fig. 1A–D,G,I,J). The expression of *tlr5a* and *clra* in response to bacterin was more strongly induced (6- to 32-fold) than *irf5* (1.3-fold), *irf7b* (up to 1.8-fold), and *tbk1* (1.4-fold).

The qPCR results of eight transcripts encoding cytokines or involved in cytokine mediated pathways are shown in Fig. 2. All eight genes [*il1b*, *tnfa*, *il8a* (fish fed CpG diet only), *il1r1*, *il10rb*, *ifng*, *irf9*, *cxcr3*] had significant induction by pIC injection when compared with the PBS-injected salmon fed the respective diet (Fig. 2). The expression of *il1b* and *ifng* in response to pIC was more strongly induced (8.7- to 27.4-fold) than the other six genes (1.5- to 4.0-fold). Seven transcripts (*il1b*, *tnfa*, *il8a*, *il10rb*, *ifng*, *irf9*, *cxcr3*) studied within this category were significantly induced by bacterin injection compared with the PBS-injected salmon fed respective diet (Fig. 2A–C, E-H). Of these genes, *il1b* showed the highest induction by bacterin (up to 20.8-fold).

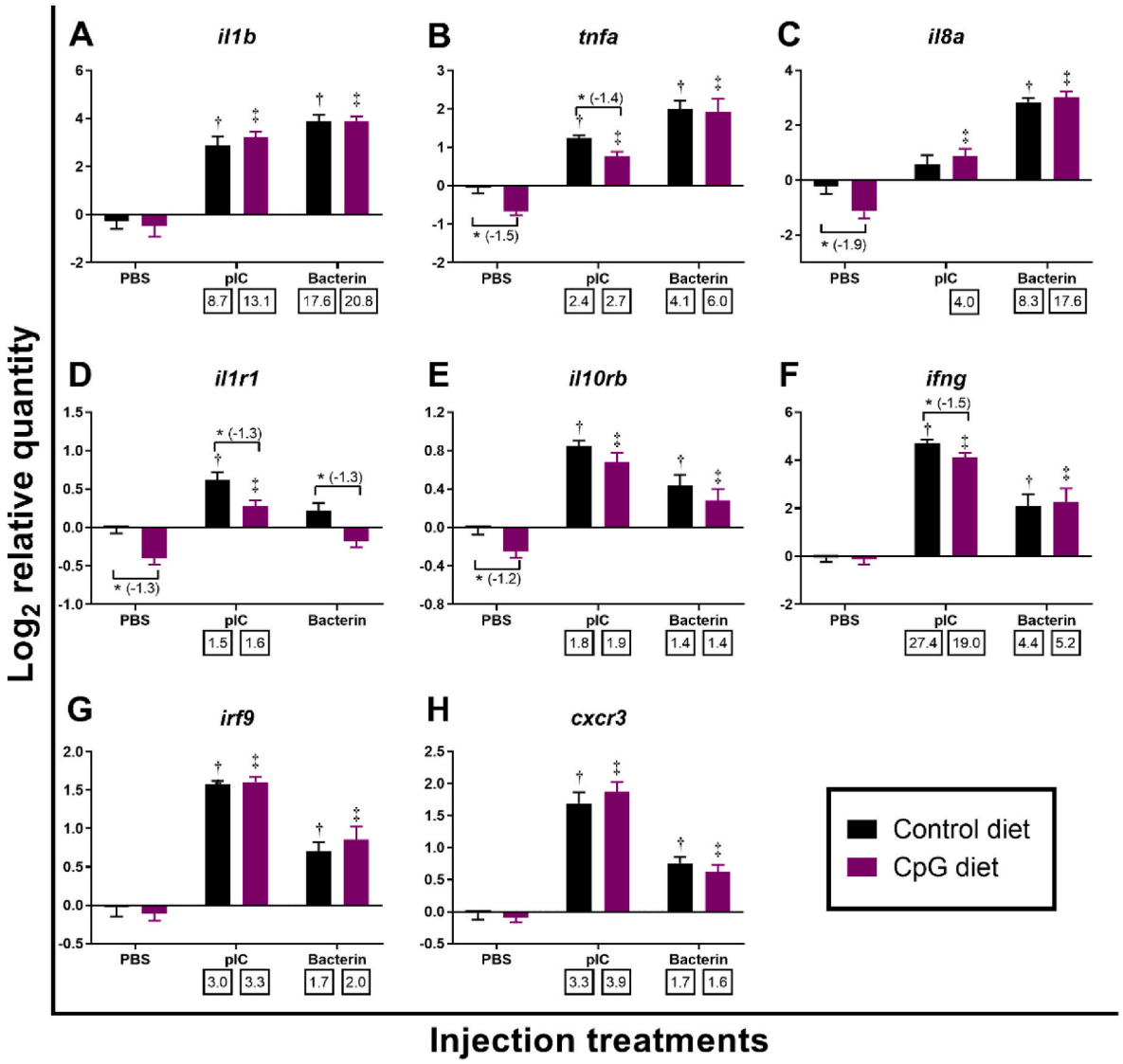

Fig. 2. qPCR analyses of transcripts encoding cytokines or involved in cytokine mediated pathways (n = 8–9). An asterisk (*) represents a significant difference between diets in each injection treatment ($p < 0.05$) with fold-change given in brackets. Fold-change between diets was calculated as 2^{A-B}, where A is the mean of $\log_2$ RQ from the CpG fed group, and B is the mean of $\log_2$ RQ from the injection-matched control fed group. For down-regulated mRNAs, fold-change values were inverted (−1/fold-change). A dagger (†) or diesis (‡) represents a significant difference between the pIC/*A. salmonicida* bacterin-injected salmon and the diet-matched PBS-injected control ($p < 0.05$) with fold-change indicated below the x-axis. Fold up-regulation or down-regulation between injections was calculated as 2^{A-B}, where A is the mean of $\log_2$ RQ from the pIC or bacterin groups, and B is the mean of $\log_2$ RQ from the diet-matched PBS group. (**A**) *interleukin 1 beta*; (**B**) *tumor necrosis factor alpha*; (**C**) *interleukin 8a*; (**D**) *interleukin-1 receptor type I*; (**E**) *interleukin-10 receptor beta chain precursor*; (**F**) *interferon gamma*; (**G**) *interferon regulatory factor 9*; (**H**) *C-X-C chemokine receptor type 3*.

The qPCR results of 11 transcripts playing key roles as immune effectors or regulators are shown in Fig. 3. Eight of these genes (*mxa*, *mxb*, *isg15a*, *campb*, *bcl6*, *mapk3*, *tnfrsf6*, *hsp90ab1*) had significant induction by pIC injection compared with the PBS-injected salmon fed respective diet (Fig. 3A–G, J). *isg15a* showed the highest induction (up to 966-fold), followed by *mxa* and *mxb* (28.7- to 39.4-fold), then *campb*, *bcl6*, *mapk3*, *tnfrsf6* and *hsp90ab1* (1.3- to 7.8-fold). The pIC injection significantly down-regulated the expression of *bnip3l* by ~1.5-fold in both diet groups (Fig. 3H). Four transcripts [*campb*, *bcl6*, *bnip3l*, *hsp90ab1* (fish fed CpG diet only)] studied within this category had significant induction by bacterin injection when compared with the PBS-injected salmon fed respective diet (Fig. 3D,E,H,J). The induction of *campb* in response to bacterin was much stronger (up to 134-fold) than that of *bcl6*, *bnip3l*, and *hsp90ab1* (1.3- to 2.9-fold).

3.2. Impact of diets on the expression of immune-relevant genes

The impact of the CpG diet on the expression of the biomarkers was investigated in both pre- and post-injection head kidney samples. This revealed that 27 cases in which the CpG diet led to significant down-regulation of a biomarker gene compared with the Control diet (Figs. 1–4). Within the pre-injection group, the CpG diet significantly reduced the transcript levels of 6 genes (−1.3 to −1.6-fold, Fig. 4C–G,L, P,AC,AD). Several genes in the post-injection groups had lower expression in the fish fed CpG diet compared with the controls (Figs. 1–3). Among the genes encoding PRRs or proteins involved in PRR signaling pathways, the CpG diet down-regulated *tlr9*, *irf5* and *sike1* (−1.2, −1.2, and −1.3-fold, respectively) in the PBS-injected fish (Fig. 1C–G,K). Within the pIC-injected group, fish fed the CpG diet had a significantly lower transcript level of *irf7a* (−1.3-fold) than those fed the Control diet (Fig. 1H). In bacterin-injected salmon, the expression level of *sike1* was

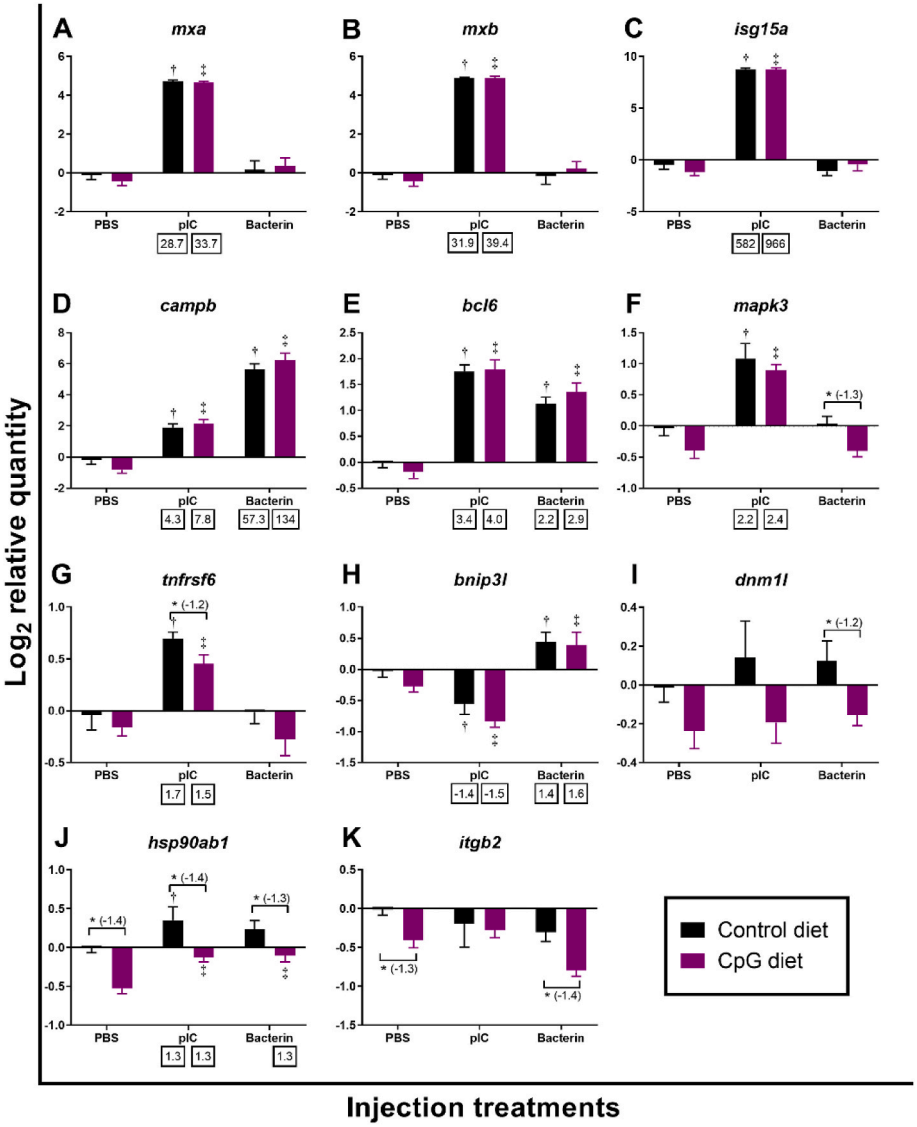

Fig. 3. qPCR analyses of transcripts encoding immune effectors and regulators (n = 8–9). Average $\log_2$ RQs with SE bars are plotted. An asterisk (*) represents a significant difference between diets in each injection treatment ($p < 0.05$) with fold-change given in brackets. Fold-change between diets was calculated as 2^{A-B}, where A is the mean of $\log_2$ RQ from the CpG fed group, and B is the mean of $\log_2$ RQ from the injection-matched control fed group. For down-regulated mRNAs, fold-change values were inverted (−1/fold-change). A dagger (†) or diesis (‡) represents a significant difference between the pIC/*A. salmonicida* bacterin-injected salmon and the diet-matched PBS-injected control ($p < 0.05$) with fold-change indicated below the x-axis. Fold up-regulation or down-regulation between injections was calculated as 2^{A-B}, where A is the mean of $\log_2$ RQ from the pIC or bacterin groups, and B is the mean of $\log_2$ RQ from the diet-matched PBS group. (**A**) *interferon-induced GTP-binding protein Mx a*; (**B**) *interferon-induced GTP-binding protein Mx b*; (**C**) *interferon stimulated gene 15a*; (**D**) *cathelicidin antimicrobial peptide b*; (**E**) *B-cell lymphoma 6 protein homolog*; (**F**) *MAP kinase-activated protein kinase 3*; (**G**) *tumor necrosis factor receptor superfamily member 6 precursor*; (**H**) *BCL2/adenovirus E1B interacting protein 3-like*; (**I**) *dynamin-1-like protein*; (**J**) *heat shock protein HSP 90*-beta; (**K**) *integrin beta-2*.

significantly lower (−1.4-fold) in fish fed the CpG diet than those fed the Control diet (Fig. 1K).

Among the transcripts encoding cytokines or other proteins involved in cytokine-mediated pathways, the PBS-injected fish fed CpG diet had significantly lower expression of *tnfa*, *il8a*, *il1r1* and *il10rb* (−1.5, −1.9, −1.3, −1.2-fold, respectively) than those fed the Control diet (Fig. 2B–E). In the pIC-injected salmon, three transcripts (*tnfa*, *il1r1* and *ifng*) were significantly down-regulated by the CpG diet (−1.4, −1.3, −1.5-fold, respectively) compared with the fish fed the Control diet (Fig. 2B–D,F). Within the bacterin-injected fish, only *il1r1* had significantly lower expression (−1.3-fold) in the fish fed CpG diet than those fed the Control diet (Fig. 2D). As for genes playing key roles as immune effectors or regulators, *hsp90ab1* and *itb2* showed lower expression (−1.4 and −1.3-fold, respectively) in PBS-injected fish fed CpG diet compared to their counterparts fed the Control diet (Fig. 3J and K). In pIC-injected fish, dietary CpG resulted in lower transcript expression of *tnfrsf6* and *hsp90ab1* (−1.2 and −1.4-fold, respectively) compared with the Control diet (Fig. 3G–J). Bacterin-injected fish fed the CpG diet showed lower levels of *mapk3*, *dnm1l*, *hsp90ab1* and *itb2* (−1.3, −1.2, −1.3, −1.4-fold, respectively) than those fed the Control diet (Fig. 3F–I, J,K).

3.3. Treatment comparisons by multivariate statistical analyses

For the pre-injection samples, the PCoA was able to segregate the two dietary groups, although relatively large variations were observed within each group (Fig. 5A). PCO1 and PCO2 accounted for 32.5 and 19.5 % of the variability, respectively. PCoA vectors (with $r \geq 0.7$) showed that fish fed the Control diet (pre-injection) were associated with several genes, including *il1r1*, *sike1*, *irf5*, *dnm1l*, *itgb2* and *il10rb*.

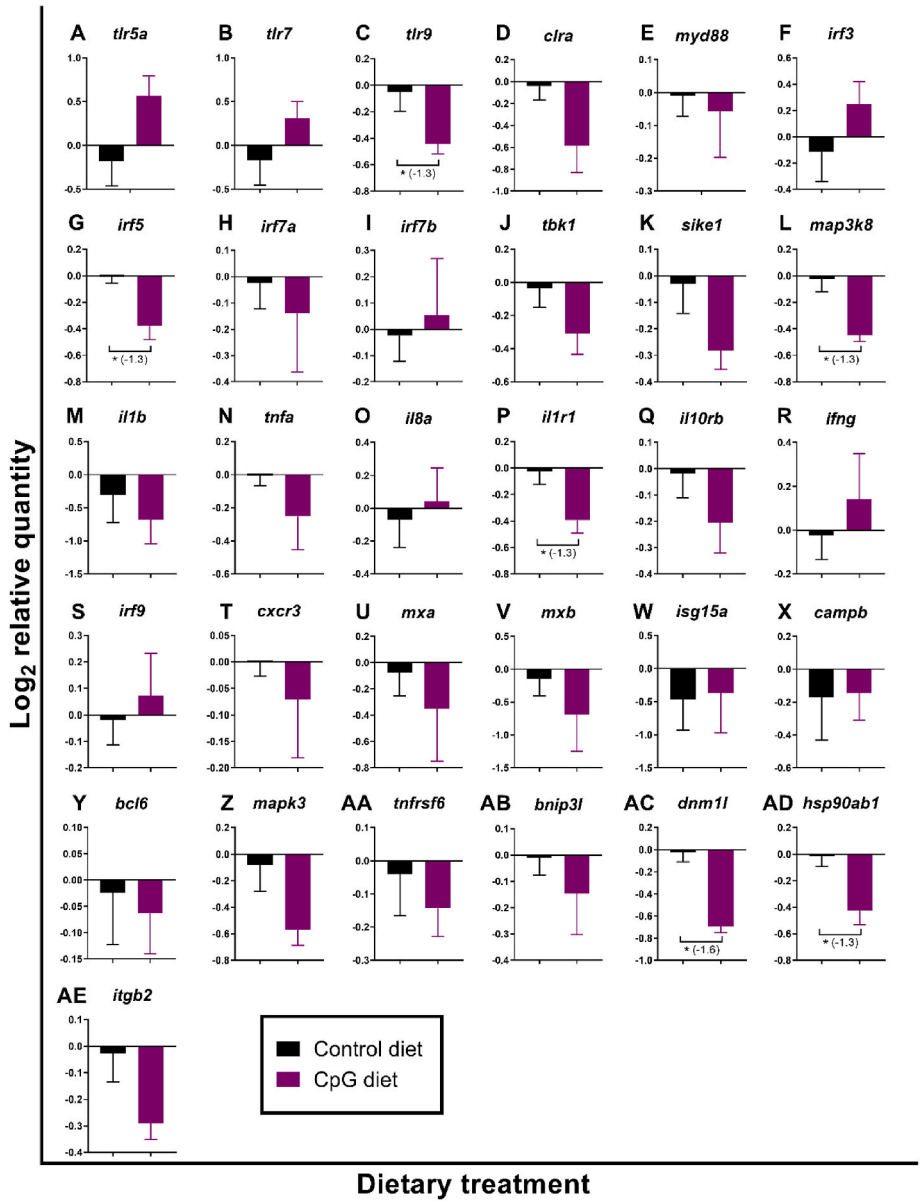

Fig. 4. qPCR analyses of basal expression (pre-injection samples) of candidate mRNAs (n = 7–8). Average $\log_2$ RQs with SE bars are plotted. An asterisk (*) indicates a significant difference between diets for a given miRNA ($p < 0.05$) with fold-change given in brackets. For qPCR fold-change calculation, overall fold up-regulation was calculated as 2^{A-B}, where A is the mean of $\log_2$ RQ from the CpG fed group, and B is the mean of $\log_2$ RQ from the control fed group. For down-regulated mRNAs, fold-change values were inverted (−1/fold-change).

PERMANOVA was conducted in order to quantify the differences among samples from fish fed different diets. The results showed that the comparisons between diets within the pre-injection group were significant based on the expression of all the qPCR-analyzed transcripts (Supplemental Table S2). As illustrated by SIMPER analysis, the comparison of mRNA expression between fish fed control and CpG diets within the pre-injection group was the most dissimilar (average dissimilarity = 28.3 %), with 12 transcripts (e.g., *dnm1l*, *mxa*, *il10rb*, *mapk3*, *isg15a*, *tnfa*, *irf9*, *il1r1*) as the top 50 % contributing variables to this dissimilarity (Supplemental Table S2).

For the post-injection groups, the PCoA was able to segregate the different injection treatments (Fig. 5B). PCO1 and PCO2 accounted for 61.6 and 18.4 % of the variability, respectively. PCoA vectors (with $r \geq 0.7$) showed that pIC-injected fish were associated with a number of genes, including *mxb*, *irf3*, *irf5*, *irf7a*, *isg15a*, *tlr7*, *tlr9* and *mapk3*, while bacterin-injected fish were associated with a different set of genes including *bnip3l*, *clra*, *campb*, *tlr5a* and *il8a*. The PERMANOVA detected overall transcript expression differences between dietary groups for the PBS- and pIC-injected fish (Supplemental Table S2). The bacterin-injected fish showed a trend (p = 0.058) towards significant diet effects. Additionally, the multivariate comparison between fish fed Control and CpG diets was the most dissimilar within the PBS-injected group (average dissimilarity = 23.7 %), followed by the bacterin- (average dissimilarity = 16.7 %) and pIC injected groups (average dissimilarity = 11.4 %). *sike1*, *dnm1l*, *il1rl*, *hsp90ab1*, and *mapk3* were common contributing variables to all dissimilarities concerning the three post-injection group comparisons (Supplemental Table S2). Finally, *il1r1*, *mapk3*, and *dnm1l* were common contributing variables to all four pairwise dissimilarities between dietary groups (pre-injection, PBS-, pIC- and bacterin-injected groups).

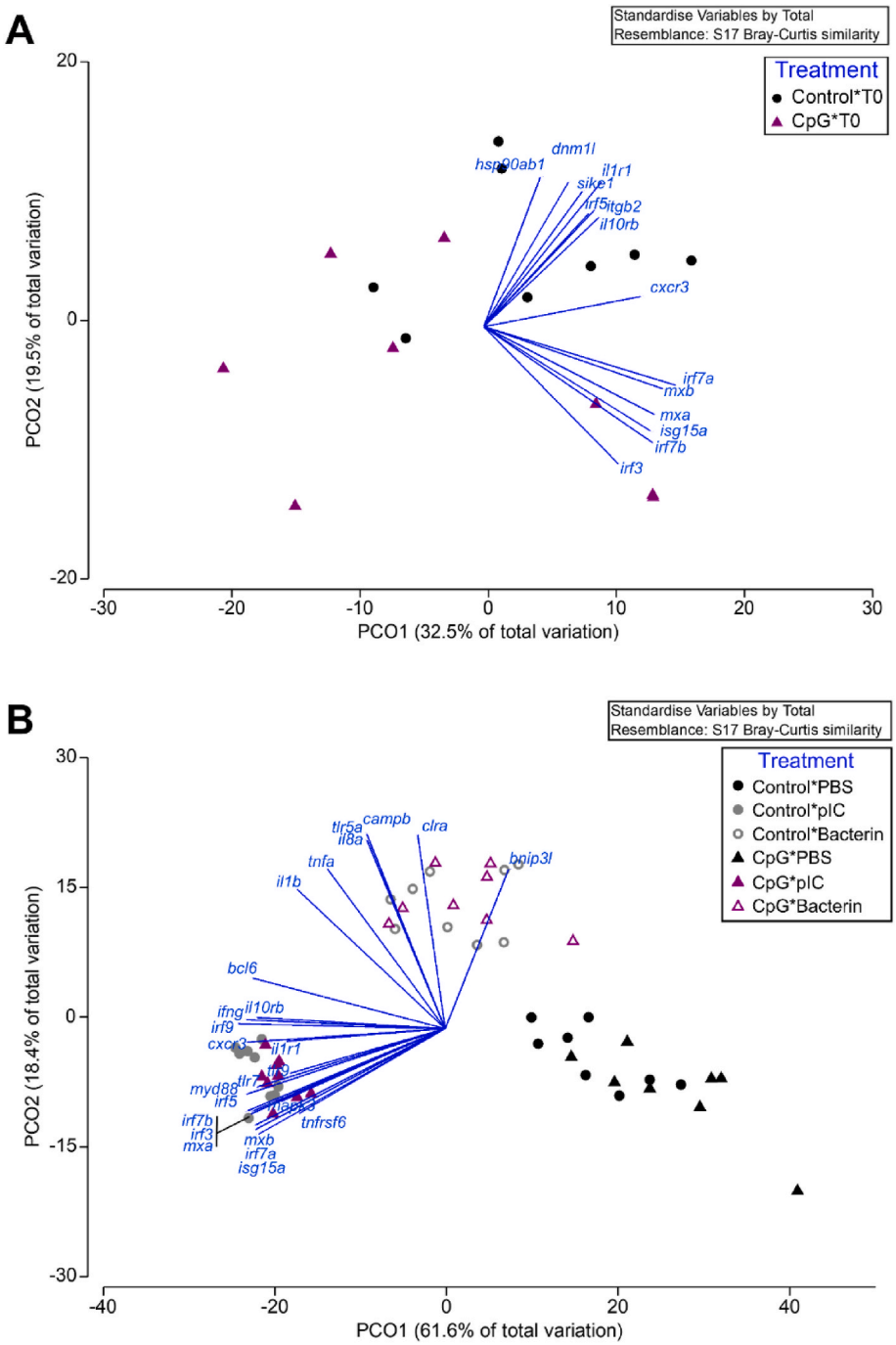

Fig. 5. Principal coordinate analyses (PCoA) of qPCR-analyzed mRNAs (RQ values) in pre-injection samples (**A**) and 24 h post-injection head kidney samples (**B**). Only vectors with Pearson correlation coefficients >0.7 are shown.

3.4. Comparisons between expression of pIC/bacterin-responsive miRNAs and their predicted target genes

The simplest model for the interaction between a miRNA and its target predicts a negative correlation between the change in their abundances; for example, if the abundance of the miRNA increases, the abundance of the target transcript decreases. Assuming that the 26 genes are true targets of the pIC/bacterin-responsive miRNAs from Xue et al. [1], this model could be directly tested, as the same samples were analyzed in both studies (i.e., present and Xue et al. [1]). Therefore, the qPCR results of miRNAs identified by Xue et al. [1] as responsive to injection treatment (pIC/bacterin *vs* PBS) and diet (CpG *vs* Control) were compared with the current mRNA expression data from their predicted targets. The comparison revealed nine cases of opposite regulation (i.e. opposite directions of fold-change) between diet-responsive miRNA and mRNA within PBS-injected fish (n = 9; e.g. *il1r1* with miR-novel-16-5p and miR-725-5p, *il10rb* with miR-29b-2-5p and miR-221-5p) (Table 2; Supplemental Table S3). Within the bacterin-injected group, the analysis revealed four cases of opposite regulations (e.g. *itgb2* with miR-29b-2-5p, *sike1* with miR-146a-1-2-3p) between putative miRNA-target pairs (Table 2; Supplemental Table S3). However, within the pre-injection group, there were five cases of regulation at same direction (i.e. same direction of fold-change) between diet-responsive miRNA and mRNA (e.g. *tlr9*, *irf5* and *dnm1l* with miR-462a-3p) (Table 2; Supplemental Table S3). It is also worth noting that no diet-responsive miRNAs were identified in the pIC-injected group [1];

Table 2
Fold-changes of diet-responsive miRNAs and their predicted target genes at different injection treatments[1].

Significant diet-responsive miRNAs	Fold-change (CpG vs Control)	Predicted targets with significant fold-changes					
		Predicted targets	Fold-change (CpG vs Control)	Predicted targets	Fold-change (CpG vs Control)	Predicted targets	Fold-change (CpG vs Control)
Pre-injection							
miR-462a-3p	−1.5	*tlr9*	−1.3	*irf5*	−1.3	*dnm1l*	−1.6
miR-192a-5p	−1.4	*map3k8*	−1.3				
miR-novel-16-5p	−1.2	*il1r1*	−1.3				
PBS-injected							
miR-29b-2-5p	1.2	*il10rb*	−1.2	*itgb2*	−1.3		
miR-221-5p	1.3	*il10rb*	−1.2				
miR-462a-3p	1.3	*tlr9*	−1.2	*irf5*	−1.2		
miR-192a-5p	1.5	*tnfa*	−1.5				
miR-194a-5p	1.6	*il8a*	−1.9				
miR-725-5p	1.7	*il1r1*	−1.3				
miR-novel-16-5p	1.4	*il1r1*	−1.3				
pIC-injected							
N/A							
Bacterin-injected							
miR-29b-2-5p	1.6	*mapk3*	−1.3	*itgb2*	−1.4		
miR-146a-1-2-3p	1.8	*sike1*	−1.4				
miR-725-5p	1.6	*il1r1*	−1.3				

[1]Fold-changes listed for miRNAs derived from previous study [1], while fold-changes of predicted target genes between diets among different injection treatments were obtained from the current study. While this table only includes genes that are predicted targets of miRNA with a significant fold-change between diets ($p < 0.05$), a full version of this table containing fold-changes of diet-responsive miRNAs and their predicted target genes at different injection treatments can be found in Supplemental Table S3. N/A = not available.

therefore, relationships between putative miRNA-target pairs in this treatment group cannot be inferred.

The results from comparing the changes in transcript levels of miRNAs and predicted targets following pIC and *A. salmonicida* bacterin treatments are given in Supplemental Table S4. In many cases where significant changes occurred in both miRNA and mRNA, they both exhibited an increase in expression levels. Only a few cases revealed opposite expression changes, as expected if there was a negative correlation between a miRNA and its putative target gene (e.g. *bnip3l* with miR-29b in pIC treatment within Control diet; *ifng* with miR-727a-3p in bacterin treatment within CpG diet).

4. Discussion

4.1. Effects of pIC and A. salmonicida bacterin injection treatments on the expression of immune-relevant transcripts

One of the goals for the current study was to study the influence of viral mimic pIC and *A. salmonicida* bacterin stimulations on the expression of the selected immune-relevant genes. The results showed that pIC and bacterin stimulation changed the expression of several transcripts encoding PRRs or proteins involved in PRR signaling pathways. Tlr5 is generally thought to recognize flagellin found in the flagellar structure of many bacteria, including *A. salmonicida* [43,44]. The current qPCR analysis showed that head kidney *tlr5a* was induced by both pIC and *A. salmonicida* bacterin injections. Interestingly, the Tlr5 of Grass carp (*Ctenopharyngodon idella*) has been documented to engage in viral recognition and exhibit binding affinity to pIC [45]. Also, infection studies in Atlantic salmon involving non-motile bacteria (e.g. *R. salmoninarum* and *P. salmonis*) showed activation of *tlr5a* in the infected animals [40,46]. We hypothesize that Atlantic salmon *tlr5a* might have evolved to acquire additional immune functions (e.g. viral recognition) compared to the mammalian orthologue. Mammalian TLR7 and TLR9, known as the endosomal PRRs, are responsible for recognizing ssRNA viruses and CpG-rich bacterial DNA/dsDNA viruses, respectively [47,48]. The up-regulation of both *tlr7* and *tlr9* by pIC in the current study is similar to that reported in Atlantic salmon macrophage-like cells exposed to pIC [49]. Skjæveland et al. [50] found that Atlantic salmon *tlr9* can be induced by recombinant trout Ifng (interferon gamma). This suggests that the up-regulation of *tlr9* in pIC-injected fish in the current study may be associated with a positive feedback loop resulting from the induction of *ifng* by pIC. Besides TLRs, we also found up-regulation of a transcript encoding C-type lectin receptor (i.e. *clra*) only in salmon injected with bacterin; it functions as a PRR and recognizes carbohydrate patterns present on the surface of microorganisms [51]. Similarly, in Soanes et al. [52], Atlantic salmon infected with live *A. salmonicida* had increased hepatic *clra* expression compared with the healthy control fish.

In the current study, the up-regulation of *myd88* in the pIC-injected salmon is consistent with increased expression of *tlr7* and *tlr9*. MyD88 is a signal transducer activated following detection of PAMPs by these endosomal PRRs, which results in the induction of transcription factors such as NFKBs and IRFs and innate immune responses [49,53]. Unlike *tlr7*, *tlr9* and *myd88,* Atlantic salmon *tlr5a* showed induction in response to both pIC and *A. salmonicida*. Mammalian TLR5 which is a cytoplasmic PRR and has also been shown to utilize the MyD88-dependent pathway [44]. The lack of *myd88* response to *A. salmonicida* bacterin found herein may suggest that the expression of Atlantic salmon *myd88* is induced through transcription factors activated by antiviral, but not antibacterial, responses. In agreement with previous work on Atlantic salmon macrophages and head kidneys after pIC stimulation [2,49], members of IRF family (i.e. *irf3*, *irf5*, *irf7a* and *irf7b*) were up-regulated by pIC in the current study. IRF3 and IRF7 are the primary family members involved in regulating the type I IFN response to viral infection, promoting the production of IFNs and interferon-stimulated genes (ISGs) [54]. The increased expression of *irf3*, *irf7a*, and *irf7b* by pIC aligns with the upregulation of myd88, as shown in a previous study where Myd88 was found to interact with Irf3 and Irf7a/b and modulate the IRF-induced IFN response in Atlantic salmon [55]. In addition to IRF3 and IRF7, IRF5 has also been shown to play a key role in retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) and mitochondrial antiviral-signalling protein (MAVS)-mediated type I IFN expression [56]. We observed significant up-regulation (1.3- to 1.8-fold) of *irf5* and *irf7b* by bacterin in salmon head kidney. Similar to our results, previous studies also reported the up-regulation of *irf5* and *irf7* by LPS in common carp (*Cyprinus carpio*) head kidney leukocytes and large yellow croaker (*Larimichthys crocea*) liver, respectively [57,58]. Further, the slight up-regulation of *irf7b* found in fish injected with bacterin in the current

study can be related to the increased expression of *tbk1* (1.4-fold in bacterin-injected group) as mammalian TBK1 has been shown to be a molecular bridge, linking the TLR and RLR signals to activate IRF3- and IRF7-mediated type I IFN response [59]. It is worth noting that the differential regulation of *irf7* paralogues (i.e. bacterin responsiveness of *irf7b* only; stronger induction of *irf7b* by pIC) suggests that these paralogues have undergone regulatory and potentially functional divergence.

Upon ligand recognition, TLR pathways activate signaling cascades that result in the induction of IFNs and cytokines, essential mediators of the inflammatory response [48]. Il1b and Tnfa (alias: IL-1β and TNF-α) are pro-inflammatory cytokines involved in the activation of a wide range of genes expressed during inflammation [60]. Il1b is predominantly produced by monocytes and macrophages, and participates in the regulation of phagocytic activity, macrophage proliferation and leukocyte migration [61]. Tnfa displays overlapping functions with Il1b; however, it also possesses the ability to induce apoptosis and necrosis [62]. Il8 (alias: Cxcl8) functions as a chemotactic factor by recruiting specific subsets of leukocytes (primarily neutrophils) to the site of infection [63]. We observed high induction of *il1b*, *il8a* and *tnfa* in fish injected with bacterin, suggesting that *A. salmonicida* bacterin can cause a strong inflammatory response. This has been observed both in intraperitoneal injections of live *A. salmonicida*, as well as isolated macrophage responses to *A. salmonicida* across multiple species [64–66]. Il1r1, a receptor of Il1b, can mediate Il1b-dependent activation of NF-kappa-B, MAPK and other pathways [67]. We found an up-regulation of *il1r1* in the salmon head kidney in response to pIC, but it was not responsive to bacterin. Interestingly, miiuy croaker (*Miichthys miiuy*) *il1r1* was found to be induced by both LPS and pIC [68]. Moreover, the expression of IL10RB protein is essential for the signal transduction of IL10, which is a key anti-inflammatory cytokine [69]. In the present study, the up-regulation of *il10rb* in both pIC- and bacterin-injected Atlantic salmon may be necessary to prevent pathological inflammation.

The up-regulation of *ifng* by pIC and bacterin in the present study suggests that a type II IFN response was activated. As shown by Caballero-Solares et al. [2] and Eslamloo et al. [49], pIC can elicit both type I and II IFN mediated antiviral responses. Irf9 plays an essential role in antiviral immunity by regulating the downstream expression of ISGs within the type I IFN response pathway [70]. The up-regulation of *irf9* by both pIC and bacterin in the current study, therefore, indicated a type I IFN response. We hypothesize that the type I IFN pathway might also play important roles in mediating immune response resulting from bacterial infection. Further, it is worth noting that pIC-injected fish in the current study exhibited robust IFN responses (both type I and II). CXCR3 is a chemokine receptor that plays an essential role in the trafficking and migration of leukocytes at sites of infection and inflammation [71]. Similar to the current findings, the expression of *cxcr3* was elevated in the kidney of large yellow croaker after *Vibrio anguillarum* challenge and pIC stimulation [72].

In the current study, we observed up-regulation of *mxa*, *mxb* and *isg15a* by pIC in salmon head kidney. This is consistent with previous reports on salmon antiviral responses [2,49]. Unlike the pIC stimulation, *A. salmonicida* bacterin injection did not result in the up-regulation of these antiviral effector genes. Therefore, it is unclear what role the type I IFN pathway plays during the bacterial infection in Atlantic salmon. Camp (i.e. Cathelicidin) is a well-known antimicrobial peptide that was found to be important in the fight against bacterial invasion in fish [73]. The up-regulation of *campb* in the bacterin-injected fish was expected as it was previously shown to be an excellent *A. salmonicida*-inducible biomarker [74].

Induction or suppression of apoptosis because of host-pathogen interaction may play a vital role in overall outcome of infection. The present study analyzed a number of genes related to the apoptotic pathways (i.e. *bcl6*, *mapk3*, *tnfrsf6*, *bnip3l*) [75–79]. Of these genes, *bcl6* was induced by both pIC and bacterin injections, whereas *mapk3* and *tnfrsf6* were only induced by pIC. Interestingly, opposite expression responses of *bnip3l* to pIC (down-regulation) and bacterin (up-regulation) were observed in the current study. Our previous work found that Atlantic salmon *bnip3l* to be suppressed following *P. salmonis* infection [40]. Collectively, the current results suggest that pIC and *A. salmonicida* bacterin stimulations modulate the apoptotic pathways in Atlantic salmon.

Furthermore, HSPs (Heat Shock Proteins) not only function as molecular chaperones, but also likely play essential roles in modulation of immune system (e.g. antigen presentation) [80–83]. A slight up-regulation of *hsp90ab1* seen with salmon injected with pIC or bacterin in the current study provides some evidence of its role in immune response and/or disease defense in Atlantic salmon.

4.2. Impact of diets on the expression of immune-relevant genes

CpG ODNs found in bacterial and some viral genomes have been shown to be important immunostimulants that can enhance fish immunity and confer protection from pathogen invasion [84]. Recognition of CpG ODNs by host PRRs occurs specifically through TLR9, resulting in the activation of antiviral and antibacterial cell signaling [16,17,85]. Our previous work revealed that dietary CpG ODN 1668 at an inclusion level of 10 mg kg^{-1} feed can modulate miRNA biomarkers associated with immune responses in Atlantic salmon [1]. Importantly, all genes significantly modulated by diet had lower expression in fish fed the CpG diet regardless of injection treatments. Unlike the miRNA expression profiles, where the biggest difference among diets was found in bacterin-injected fish [1], the overall expression differences in qPCR-analyzed transcripts between diets were most dissimilar among pre-injection and PBS-injected fish based on the current SIMPER analyses.

Among transcripts encoding PRRs or proteins involved in PRR signaling pathways, *tlr9* and *irf5* had lower expression in both pre- and PBS-injected fish fed the CpG diet. As discussed earlier, mammalian TLR9 is responsible for recognizing CpG-rich bacterial DNA/viral dsDNA, respectively [47]. Studies on the regulation of fish *tlr9* after stimulation with CpG ODNs via IP-injection are conflicting since both up- and down-regulation of *tlr9* expression have been described, which may reflect different classes of CpG ODN or time points used in these studies [19,20,85,86]. Purcell et al. [22] showed that Atlantic salmon fed diet containing CpG ODN 1668 had significantly decreased *tlr9* expression over time in both skin and spleen. Alongside the current results, these findings suggest that *tlr9* may be under a negative feedback loop control in Atlantic salmon. The down-regulation of *irf5* (among pre-injection and PBS-injected groups) and *irf7a* (among pIC-injected groups) by CpG diet indicates that the RLR- and MAVS-mediated type I IFN response may be attenuated by dietary CpG. Furthermore, mammalian SIKE1 interacts with IKBKE (i.e. Inhibitor of nuclear factor kappa b kinase subunit epsilon) and TBK1, thus playing an inhibitory role in virus-triggered TLR3-dependent IFN activation pathways [87]. In the present study, down-regulation of *sike1* seen in PBS- and bacterin-injected fish fed CpG diet once again indicated that dietary CpG might modulate IFN-related pathways. It has been shown in mammals that MAP3K8, a serine-threonine kinase, is critical in innate immunity, transducing signals from TLRs to regulate TNFA and IL1B production [88]. The down-regulation of *map3k8* in pre-injected fish fed CpG diet found in the current study suggests that dietary CpG could potentially influence Map3k8 signaling and thereby regulate *tnfa* and *il1b* in Atlantic salmon.

Among the transcripts encoding cytokines or other proteins involved in cytokine-mediated pathways, *il1r1* was consistently down-regulated in fish fed CpG from all injection treatment groups. We hypothesize that dietary CpG is able to modulate the Il1b-dependent activation of NF-kappa-B and MAPK pathways. In addition, the current qPCR study showed that the expression of three important inflammatory markers (i.e. *il8a*, *il10rb*, *tnfa*) was inhibited in PBS- and pIC-injected (*tnfa* only) fish fed CpG diet, suggesting a lesser inflammatory response in these

animals. These results could also indicate that fish-fed the CpG diet might be able to resolve inflammatory responses more quickly after a generalized stress event (e.g. PBS injection) or pIC stimulation. Moreover, the down-regulation of *ifng* in pIC-injected salmon fed CpG diet found in the present study suggests that the type II IFN-mediated antiviral response may be altered. Since *ifng* also plays regulatory roles in both innate and adaptive immunity (e.g. activating macrophages, enhancing antigen presentation), it is reasonable to speculate that CpG-driven immune modulation may have an impact on the adaptive responses in Atlantic salmon. This hypothesis gains additional support from a prior investigation into the inclusion of CpG in salmon diets [22], which suggested that CpG not only boosts innate responses to sea lice but also provides further stimulation to adaptive responses.

Among the transcripts playing critical roles as immune effectors or regulators, the transcript levels of *hsp90ab1* and *itgb2* were consistently down-regulated in fish fed CpG from all injection treatment groups (except *itgb2* among pIC-injected fish). As discussed earlier, *hsp90ab1* may play additional roles in immune responses (e.g. antigen presentation). Moreover, in mammalian species as well as zebrafish, adhesion molecules of the β_2 integrin family (e.g. ITGB2, also referred to as CD18) are necessary for neutrophil recruitment to sites of inflammation [89]. The qPCR results on *hsp90ab1* and *itgb2* suggest that dietary CpG might modulate other immune system functions in Atlantic salmon, such as antigen presentation and neutrophil recruitment. Lastly, the down-regulation of apoptosis-relevant genes (i.e. *mapk3*, *dnm1l*, *tnfrsf6*) among pre-, pIC- and/or bacterin-injected fish fed CpG diet indicates that the apoptotic pathways may be suppressed by dietary CpG in Atlantic salmon. As indicated by several studies of bacterial [90,91] and viral diseases [92,93] in salmonids, strong up-regulation of innate immune mechanisms and cellular stress are often linked to increased pathology and other adverse outcomes. Therefore, it can be hypothesized that the reduced basal expression of genes associated with innate immunity and apoptosis pathways and attenuated antibacterial and antiviral responses in fish fed the CpG diet could prove advantageous by mitigating the immune response-driven damage posed by pathogen challenge.

4.3. Comparisons of impacts of pIC and A. salmonicida bacterin treatments and dietary CpG on immune-responsive miRNAs and predicted mRNA target levels

miRNAs have been well-documented to cause transcriptional degradation and/or translational repression [24,94]. It has also been previously shown that the miRNA-mRNA regulatory network is very complex [95]. A single miRNA can regulate multiple target mRNAs, while a given mRNA can be targeted by various miRNAs [96]. In our study of CpG diets and miRNA expression [1], we showed that many miRNAs responded to pIC and bacterin treatment and that CpG supplementation generally suppressed basal expression (i.e. in pre-injection samples) of many of these pathogen-responsive miRNAs (e.g. miR-192a-5p, miR-462a-3p, miR-181a-5-3p). In the post-injection groups, dietary CpG had significant impacts on the miRNA expression in both PBS and bacterin-injected fish with several miRNAs (e.g., miR-181a-5-3p, miR-221-5p, miR-29b-2-5p) showing higher expression in fish fed the CpG-containing diet. To complement this study on the impact of dietary CpG on miRNA expression in Atlantic salmon [1], we explored the correlation between the expression changes in the pIC/-bacterin responsive-miRNAs and their predicted target mRNAs analyzed in the present study. This could provide insight on whether the mechanism for regulation is mainly by degradation of the target or if other processes, like interfering with the translation of the target transcript, may be the more important regulatory mechanism. The general finding when comparing direction changes for immunogen-responsive miRNAs and their predicted target genes within a given dietary group (i.e. Supplemental Table S4) was that the transcript levels of both the predicted target and the miRNAs increased. Thus, a simple model with opposite changes in transcript levels of a miRNA and its target was not supported. In contrast, a high number of opposite regulations between miRNA and mRNA was identified within PBS-injected fish (i.e. Table 2). In the current study, PBS-injected group was used to factor the handling stress (e.g. netting, light anesthesia, and injection-associated stress) caused during immune stimulations. The opposite regulations found between miRNAs and mRNAs within PBS-injected fish could support a regulatory mechanism where the diet-driven changes in miRNA and immune-related transcript levels similarly affect, and together possibly enhance, the modulation of immune pathways. Since these putative target genes (e.g. *il1r1*, *il8a*, *il10rb*) play key roles in modulating many important immune pathways, including the activation of NF-kappa-B pathway [67], the opposite regulations between miRNAs and mRNAs found in PBS-injected fish in the current study may further support the notion that miRNAs fine-tune the expression of stress- and/or immune-responsive transcripts.

There were a few instances where the regulation occurred in the same direction between miRNA and mRNA expression representing putative miRNA-target pairs (e.g. *tlr9*, *irf5* and *dnm1l* with miR-462a-3p in pre-injection fish). As noted by others [29,97,98], same direction of regulation between predicted miRNA-target pairs is common. This observation agrees with a negative regulatory mechanism that inhibits the targets at the translational level. A study in zebrafish suggests that this is the major mechanism of RNA silencing in teleost fish [99], but any studies of Argonaut proteins and their slicing capacity in salmonids have not so far been conducted. A same direction of regulation between a miRNA-target pair can also be explained by the feed-forward loop mechanism that regulates miRNA and its target mRNA in the same direction [97]. Andreassen and Høyheim [23] proposed a similar miRNA and mRNA interaction model (e.g. expression dynamics) for miRNAs associated with immune responses in teleost fish. Under this model, the increase in miRNA expression along with the increase in target (i.e. mRNA) expression is needed to ensure a balanced immune response. Finally, there may also be a large number of false positives among targets predicted by the commonly used *in silico* methods. In fact, the miRNAs associated with pIC and bacterin responses in Xue et al. [1] were each predicted as targeting from 2 to 21 immune relevant genes. Predictions are based on the nucleotide sequence of the 3′UTRs and the fact that all miRNAs and predicted transcripts investigated in the current study are co-expressed. However, secondary structures and internal loops within transcripts may inhibit miRNA-transcript interaction. Such falsely predicted miRNA/target genes are not expected to show any correlation [100]. In summary, the current expression comparisons between miRNAs and their predicted targets could not provide further evidence that they were true targets or support a model where a target transcript is degraded. Rather, the comparisons agreed with previous observations that the function of a given miRNA is complex and can usually not be inferred from its interaction with one single transcript. Consequently, it indicates that the use of the immune response genes and the previously identified immune response miRNAs as biomarkers would provide complementary information on the immune status and effect of feed rather than overlapping information.

5. Conclusions

To expand our previously published work on immune-relevant miRNAs in Atlantic salmon, the present study explored the impact of viral mimic pIC and *A. salmonicida* bacterin stimulations on the expression of immune-relevant biomarkers in Atlantic salmon. The current qPCR data showed that pIC and/or bacterin stimulations significantly modulated many predicted target genes of miRNAs from our previous study which are involved in various immune pathways. Immunogen-specific expression patterns were also observed. For example, high induction of pro-inflammatory genes (e.g. *il1b*, *il8a* and *tnfa*) in bacterin-injected fish suggests that *A. salmonicida* bacterin induced a strong inflammatory response. On the other hand, pIC-

injected fish in the current study showed robust IFN responses (both type I and II). Significant modulations of immune-relevant transcripts by dietary CpG were also evident, with higher impacts seen among pre-injection and PBS-injected fish based on the current results. Surprisingly, all genes that showed significant modulation by diet had lower expression in CpG diet fed fish regardless of injection treatments. In the current study, we observed both opposing and concordant regulation among various pairs of miRNAs and their predicted targets, indicating a complex nature of the miRNA-mRNA regulatory network. Future studies are needed to evaluate the effects of dietary immunostimulant CpG ODN 1668 on Atlantic salmon responses to live viral and bacterial infections.

CRediT authorship contribution statement

Xi Xue: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft. **Khalil Eslamloo:** Investigation, Data curation, Writing – review & editing. **Albert Caballero-Solares:** Investigation, Data curation, Writing – review & editing. **Tomer Katan:** Methodology, Formal analysis, Data curation, Writing – review & editing. **Navaneethaiyer Umasuthan:** Investigation, Writing – review & editing. **Richard G. Taylor:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing. **Mark D. Fast:** Conceptualization, Methodology, Writing – review & editing. **Rune Andreassen:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Matthew L. Rise:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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