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Profiling the transcriptome response of Atlantic salmon head kidney to formalin-killed *Renibacterium salmoninarum*

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ABSTRACT

Renibacterium salmoninarum is a Gram-positive, intracellular bacterial pathogen that causes Bacterial Kidney Disease (BKD) in Atlantic salmon (*Salmo salar*). The host transcriptomic response to this immune-suppressive pathogen remains poorly understood. To identify *R. salmoninarum*-responsive genes, Atlantic salmon were intraperitoneally injected with a low (5×10^5 cells/kg, Low-Rs) or high (5×10^7 cells/kg; High-Rs) dose of formalin-killed *R. salmoninarum* bacterin or phosphate-buffered saline (PBS control); head kidney samples were collected before and 24 h after injection. Using 44K microarray analysis, we identified 107 and 345 differentially expressed probes in response to *R. salmoninarum* bacterin (i.e. High-Rs vs. PBS control) by Significance Analysis of Microarrays (SAM) and Rank Products (RP), respectively. Twenty-two microarray-identified genes were subjected to qPCR assays, and 17 genes were confirmed as being significantly responsive to the bacterin. There was an up-regulation in expression of genes playing putative roles as immune receptors and antimicrobial effectors. Genes with putative roles as pathogen recognition (e.g. *clec12b* and *tlr5*) or immunoregulatory (e.g. *tnfrsf6b* and *tnfrsf11b*) receptors were up-regulated in response to *R. salmoninarum* bacterin. Also, chemokines and a chemokine receptor showed opposite regulation [up-regulation of effectors (i.e. *ccl13* and *ccl*) and down-regulation of *cxcr1*] in response to the bacterin. The present study identified and validated novel biomarker genes (e.g. *ctsl1, lipe, cldn4*, *ccny*) that can be used to assess Atlantic salmon response to *R. salmoninarum*, and will be valuable in the development of tools to combat BKD.

1. Introduction

Aquaculture has been predicted to be the main source fulfilling the future global demand for fish consumption [1,2]. Atlantic salmon (*Salmo salar*) is one of the most economically important species in marine finfish aquaculture, and its production is increasing worldwide [1,3]. However, Atlantic salmon are susceptible to several bacterial pathogens that can cause high mortalities and economic losses [4,5]. *Renibacterium salmoninarum* is a nonmotile, Gram-positive, rod-shaped bacterium causing Bacterial Kidney Disease (BKD) in salmonid species such as sockeye salmon (*Oncorhynchus nerka*), rainbow trout (*O. mykiss*) and Atlantic salmon in both freshwater and seawater environments [6]. This bacterium can be horizontally and vertically transmitted, and BKD has been reported in several countries worldwide including Canada and Chile [6,7].

Following *R. salmoninarum* infection, granulomas – white nodules containing epithelioid cells such as macrophages – develop in the haematopoietic kidney tissue and extend to other internal organs (e.g. posterior kidney and liver) of the fish [7–9]. BKD can cause up to 40% and 80% cumulative mortality in Atlantic salmon and Pacific salmonids (*Oncorhynchus* spp.), respectively [8,9]. BKD-derived mortalities are also attributed to immunosuppression by *R. salmoninarum* and, consequently, the increased susceptibility of infected fish to secondary pathogens [10]. Live-attenuated and formalin-killed *R. salmoninarum* vaccines have been reported to improve the resistance of salmonids such as Atlantic salmon to BKD [9,11]. The grave threat posed by *R. salmoninarum* to Atlantic salmon aquaculture, alongside the immunosuppressive feature of this pathogen, necessitates the studying of the Atlantic salmon response to BKD and formalin-killed *R. salmoninarum.* Previous studies showed a BKD-induced serum antibody response in Atlantic salmon and rainbow trout [12], and an *inducible nitric oxide synthase* (*inos*) transcript expression response in rainbow trout kidney [13]. Furthermore, *in vitro* studies determined the expression of inflammation-related genes of rainbow trout macrophages [14] or

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Atlantic salmon kidney (ASK) cell line [15] in response to *R. salmoninarum.* Suppressive subtractive hybridization (SSH) and qPCR-based studies were conducted to identify the genes associated with *R. salmoninarum* response in Chinook salmon (*O. tshawytscha*) [16,17]. However, the genes, biological processes and molecular pathways activated in response to live or formalin-killed *R. salmoninarum* in Atlantic salmon remained uncharacterised.

Microarray analyses can be employed to profile the transcriptome response of a species to a given stimulus, and can help to draw a comprehensive picture of the immune pathways involved [18]. For example, microarray analyses were previously used to determine the global gene expression of Atlantic salmon in response to *Piscirickettsia salmonis* [19], *Aeromonas salmonicida* [20], live *A. salmonicida* vaccine [21] and commercial vaccines (e.g. for immunization against *Yersinia ruckeri* and *Vibrio* spp.) [22,23].

The consortium for Genomic Research on All Salmonids Project (cGRASP)-designed Agilent 44K salmonid oligonucleotide microarray [24] is a powerful tool that was previously used to profile the immunological responses of Atlantic salmon and rainbow trout [25–28]. In the present study, we used this 44K salmonid microarray platform to profile the Atlantic salmon head kidney transcriptome response to formalin-killed *R. salmoninarum*. The current study aimed to identify biomarker genes and immune pathways dysregulated in response to killed *R. salmoninarum,* thus developing a better understanding of the molecular processes underlying the Atlantic salmon response to this bacterin. Further, given the high level of genomic similarity among salmonids [29], our results may be valuable in the identification of biomarkers for evaluating the immune response of other salmonid species such as rainbow trout to formalin-killed *R. salmoninarum.* Here, we found that formalin-killed *R. salmoninarum* induced the expression of genes associated with inflammation-relevant, antibacterial as well as cytokine responses, and it suppressed the expression of genes playing putative roles as a cytokine receptor and kinase regulator.

2. Materials and methods

2.1. R. salmoninarum strain and culture

R. salmoninarum (ATCC33209) was cultured in complex KDM-2 [1.0% Peptone (Difco), 0.05% Yeast (Difco), 0.1% L-cysteine HCl (Sigma-Aldrich, St. Louis, MO), 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA)] [30] at 15 °C for 4 weeks. When required, 1.5% agar (Difco) was added.

2.2. Bacterin preparation

Ten KDM-2 agar plates were inoculated with a fresh culture of *R. salmoninarum* and incubated at 15 °C for 4 weeks. The bacteria grown on the agar plates were harvested and re-suspended in phosphate-buffered saline (PBS; pH 7.2, Gibco). The bacterial cell suspension was then washed three times with PBS by centrifugation at 4200 × *g* for 10 min at 4 °C. *R. salmoninarum* cells were inactivated with 6% formaldehyde for 3 days at room temperature with gentle agitation. Formalin was removed by centrifugation at 4200 × *g* for 10 min at 4 °C. The bacterial cell pellet was re-suspended and dialysed in Slide-A-Lyzer Dialysis Cassettes (20 K MWCO, 12 ml, Thermo Fisher Scientific, Waltham, MA) in PBS for 3 days at 6 °C. The bacterin cell concentrate was quantified using Bacteria Counting Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA) for flow cytometry based on the manufacturers' instructions. Also, a BD FACS Aria II flow cytometer (BD Biosciences, San Jose, CA) and BD FACS Diva v7.0 software were used for bacterial cell quantification. The number of bacterial cells ml^{-1} of the cell suspension was calculated by dividing the number of signals in the bacterial frame by the number of signals in the microsphere frame (Supplemental Fig. S1). Formalin-killed *R. salmoninarum* bacterin was then stored at 4 °C at a concentration of 10^8 cells ml^{-1} until immunization. Inactivation of the bacteria was confirmed by sub-culturing of formalin-killed bacterin on KDM-2 agar plates. *R. salmoninarum* bacterin was diluted using PBS (Gibco) to achieve the final concentration of 5×10^7 cells ml^{-1} (high dose) and 5×10^5 cells ml^{-1} (low dose) for injection.

2.3. Bacterin injection and sampling

For this study, we used 23 Atlantic salmon [1.66 ± 0.07 kg (mean ± SE)] from a local farm that had been PIT (passive integrated transponder)-tagged and reared at the Dr. Joe Brown Aquatic Research Building (JBARB; Ocean Sciences Centre, St. John's, NL, Canada). Three weeks prior to the sampling and injection, fish were transferred to a 3000 L tank supplied by a flow-through seawater system. Fish were fed at 1.0% of their average body weight every 2 days using a commercial diet at ~10 °C and under 12-h light photoperiod during the adaptation period. Fish were fasted 24 h before injection and sampling. Fish were euthanized with an overdose of MS222 (400 mg L^{-1}; Syndel Laboratories, Vancouver, BC) before sampling; tissue samples were flash-frozen using liquid nitrogen and kept at −80 °C until RNA extraction and analyses. Five fish were euthanized and sampled for head kidneys before the remaining fish were injected. This group was used as the unstressed (pre-injected) control group (Pre-Inj; $n = 5$). The remaining 18 Atlantic salmon were lightly anaesthetised using MS222 (50 mg L^{-1}), and subsequently injected intraperitoneally with either a low (5×10^5 cells kg^{-1} wet mass; Low-Rs; $n = 6$) or high (5×10^7 cells kg^{-1} wet mass; High-Rs; $n = 6$) dose of formalin-killed *R. salmoninarum,* or PBS (PBS control; $n = 6$). The low and high doses of bacterin in the current investigation were close to the doses of live *R. salmoninarum* that were previously reported to cause varying rates of mortality in Atlantic salmon. Daly et al. [11] found that 5×10^6 *R. salmoninarum* can cause mortalities (100% after 40–50 days) in Atlantic salmon, but earlier mortalities were seen when fish were infected with 10^8 *R. salmoninarum* (100% after 15 days). Fish in all experimental groups were injected with 1 ml kg^{-1} wet mass. The injected fish were held in a 3000 L tank connected to the flow-through water system until sampling. Fish were euthanized using an overdose of MS222 (400 mg L^{-1}; Syndel Laboratories), and the head kidney samples were collected at 24 h post-injection (HPI). All procedures in this study were approved by Memorial University of Newfoundland's Institutional Animal Care Committee (protocol number: 18-01-MR), based upon the guidelines of the Canadian Council on Animal Care.

2.4. RNA extraction and purification

Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's instructions. Head kidney samples were TRIzol-lysed using RNase-Free Disposable Pellet Pestles (Thermo Fisher Scientific), and lysed samples were passed through QIAshredder (Qiagen, Hilden, Germany) homogenizer spin columns prior to the RNA extraction. To remove residual genomic DNA, RNA samples (~50 μg) were treated with 6.8 Kunitz units of DNaseI (Qiagen) for 10 min at room temperature, according to the manufacturer's instructions. DNase-treated RNAs were then purified using the RNeasy MinElute Cleanup Kit (Qiagen) based on the manufacturer's recommendations. The column-purified RNAs were quantified and checked for purity using NanoDrop spectrophotometry (ND-1000), and RNA integrity was determined by 1% agarose gel electrophoresis. The RNA samples used in this study were of high purity (i.e., A260/230 and A260/280 ratios > 1.8) and integrity (i.e., tight 18S and 28S ribosomal RNA bands).

2.5. Microarray experimental design and hybridization

Based on qPCR data obtained from the analysis of 2 well-known antibacterial genes [i.e. *hepcidin antimicrobial peptide* (*hamp*) and *cathelicidin antimicrobial peptide* (*camp*); see Fig. 6 in the Results section]

in all head kidney samples, the High-Rs group was selected for transcriptome profiling. The qPCR methods (e.g. cDNA synthesis, normaliser and PCR program) for these assays are described in section 2.8. Head kidney samples collected from 6 individuals in PBS and High-Rs group were subjected to microarray analysis (i.e. 12 samples in total). The present microarray experiment was designed following the MIAME guidelines [31], using the cGRASP-designed Agilent 44K salmonid oligonucleotide microarray [24] as described in Xue et al. [32]. Briefly, anti-sense amplified RNA (aRNA) for each individual sample was generated by *in vitro* transcription using 1 μg of DNase-treated and column-purified RNA and the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. The aRNAs were quality-checked and quantified using agarose gel electrophoresis and NanoDrop spectrophotometry, respectively. The common reference consisted of a pool from aRNA of all 12 samples (i.e. 15 μg from each sample) in this experiment. Using a standard ethanol precipitation method, 20 μg of aRNA from each experimental sample or common reference were precipitated and re-suspended in coupling buffer (Ambion). Then, the common reference was labelled with Cy3 (GE Healthcare Life Sciences, Buckinghamshire, UK), and the experimental samples were labelled with Cy5 (GE Healthcare Life Sciences) following the manufacturer's instructions. The labelled aRNA concentration and labelling efficiency were determined using the microarray feature in NanoDrop spectrophotometry software (ND-1000 v3.8.1). The labelled aRNA from each experimental sample (i.e. 825 ng) and the common reference (i.e. 825 ng) were pooled and fragmented following the manufacturer's recommendation (Agilent, Santa Clara, CA). Thereafter, each pool (i.e. an individual sample and common reference) of fragmented labelled aRNA was co-hybridized to a 44K microarray at 65 °C for 17 h with rotation (10 rpm) using an Agilent hybridization oven. Slides were washed with Gene Expression Wash Buffer 1 (Agilent) at room temperature and then Gene Expression Wash Buffer 2 (Agilent) at 37 °C using 50 ml conical centrifuge tubes and a rocking platform [VWR Rocker (Radnor, PA): speed 40, tilt 6] for 5 min. These wash buffers were supplemented with 0.5 μl ml^{-1} of 10% Triton X-102 (Agilent), as recommended by the manufacturer. Before scanning, the residual wash buffer was removed from slides by centrifuging at $200 \times g$ for 5 min at room temperature.

2.6. Microarray data acquisition and analyses

Microarray slides were scanned at 5 μm resolution using a SureScan Microarray Scanner System (Agilent) and Microarray Scan Control Software v.9.1 following the Agilent HD 2-color gene expression microarrays scan protocol. The Cy3 and Cy5 channel photomultiplier tube (PMT) settings were automatically adjusted by the Software and the raw data were saved as TIFF images. Agilent Feature Extraction Software v12.0 (Agilent) was used to extract and Loess-normalise the signal intensity data. Thereafter, using GeneSpring Software v14.9 (Agilent), the data were quality-checked so that probes with low and marginal quality, as well as absent values in more than 25% of all 12 arrays, were removed from the dataset. The missing values were imputed by GeneSpring Software. The final dataset exported from GeneSpring and subjected to the statistical analyses in this study consisted of 31,974 probes for all arrays (GEO accession number: GSE135168). Significance Analysis of Microarrays (SAM) [33] and Rank Products (RP) [34,35] were used to identify differentially expressed probes (DEP) responsive to *R. salmoninarum* bacterin in this study. SAM and RP analyses were conducted using R (version 3.4.3) and the Bioconductor packages siggenes [false discovery rate (FDR) = 10%] and RankProd [percentage of false-positives (PFP) = 10%], respectively.

The SAM- and RP-identified transcripts were re-annotated using the contigs [24] used to design the 60mer oligonucleotide probes. Blast2GO software version 5.1 (BioBam Bioinformatics S.L., Valencia, Spain) was employed to conduct the BLASTx searches of NCBI's non-redundant (nr) amino acid sequence from human and Swiss-Prot databases (E-value < 1e-5) [36–38]. Blast2GO software was used to conduct Gene Ontology (GO) term enrichment analysis (Fisher's exact test, FDR cutoff of 5%). The microarray log_2 ratios of the SAM- and RP-identified DEP and the genes selected for qPCR validation were median-centred and subjected to Pearson correlation and complete linkage hierarchical clustering using the Genesis software, version 1.8.1 (Rockville, MD) [39].

2.7. Pathway analysis

The Pathifier analysis [40] utilises pathway annotation resources (e.g. Molecular Signatures Database, MSigDB) [41] to calculate the Pathway Deregulation Score (PDS) of a given pathway in each sample. Pathifier Bioconductor package 1.16.0 implemented in R was used to compute the PDS for each pathway on microarray expression data of individual samples [40]. MSigDB version 6.1 contains 17,786 gene sets divided into 8 main collections and several sub-collections based on database types, ontology resources or positional annotation. From the normalised gene expression data (i.e. the microarray data subjected to the SAM and RP analyses), Pathifier transformed the signal values of genes into pathway-level measurements i.e. PDS ranges from 0 to 1 in which higher value of PDS reflects a higher degree of dysregulation compared to the reference (i.e. PBS control group). The normalised microarray data consisting of 10,008 genes (31,974 probes) from 12 samples (6 High-Rs and 6 PBS control) were used as input for Pathifier. The output of Pathifier analysis was each pathway/gene set per sample.

2.8. qPCR validation

A subset of microarray-identified transcripts was subjected to qPCR analysis to confirm the microarray results. We selected 16 (i.e. 15 up-regulated and 1 down-regulated) *R. salmoninarum* bacterin-responsive transcripts identified by both SAM and RP analyses. Also, 6 *R. salmoninarum* bacterin-responsive transcripts (i.e. 1 up-regulated and 5 down-regulated) identified only by RP were included in the qPCR experiment. Transcript levels of these genes of interest (GOIs) were measured in all of the collected samples from all groups (i.e. Pre-Inj, PBS, Low-Rs and High-Rs; 23 samples in total).

First-strand cDNA templates were synthesized in 20 μl reactions using 1 μg of DNaseI-treated, column-purified total RNA, 1 μl of dNTPs (10 mM each; Invitrogen), random primers (250 ng; Invitrogen) and M-MLV reverse transcriptase (200 U; Invitrogen) with the first-strand buffer (1X final concentration) and DTT (10 mM final concentration) following the manufacturer's instructions. The cDNA synthesis was performed at 37 °C for 50 min, and the reaction was then inactivated by incubation at 70 °C for 15 min.

The qPCR assays used in this study were conducted according to the MIQE guidelines [42]. All qPCR assays were performed in the ViiA 7 Real-Time PCR system (384-well format) (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) using 13 μl reactions consisting of 1X Power SYBR Green PCR Master Mix (Applied Biosystems), 50 nM of each forward and reverse primers, and the indicated cDNA quantity (see below). The qPCR assays in this study were performed in triplicate, except for the normaliser test that was conducted in duplicate. The details of qPCR reactions and program are described in Eslamloo et al., [43]. Primers used for qPCR assays were either designed using Primer3web v4.0.0 (http://primer3.wi.mit.edu) or taken from previous studies (see Table 1). Two pools were generated using cDNA of 3 individuals from High-Rs or PBS groups for primer quality control (QC) of up- and down-regulated genes, respectively. For each GOI and candidate normaliser, the performance and amplification efficiencies of primer sets were calculated using a 5-point, 3-fold serial dilution of the given cDNA template (i.e. standard curves; starting with cDNA representing 10 ng of input total RNA), as well as a no-template control. Primer QC tests were carried out in triplicate. Primer pairs that had no primer-dimer present in the no-template control, an amplicon with a single melting peak and an amplification efficiency [44] between 90

Table 1
Primers used in qPCR studies.

Gene name	GenBank accession number		Primer sequence 5′ to 3′	R^2	Amplification efficiency (%)	Amplicon size (bp)
Genes of interest						
toll-like receptor 5 (*tlr5*)	AY628755	Forward	ATCGCCCTGCAGATTTTATG	0.998	98.9	103
		Reverse	GAGCCCTCAGCGAGTTAAAG			
tumor necrosis factor receptor superfamily member 6b (*tnfrsf6b*)	EG881931	Forward	CCCAGGTCGCACCACTATAC	0.994	104.1	112
		Reverse	CATCAACTCCCCATCACAGA			
tumor necrosis factor receptor superfamily member 11b (*tnfrsf11b*)	BT049358	Forward	CTGTCCTCAGGGGTACGTGT	0.997	95.1	154
		Reverse	CTGACCAGCTTCCTCAGCTT			
C-type lectin domain family 12 member b (*clec12b*)	EG842232	Forward	GGGTATTGGATCGGTTTGAC	0.991	95.7	109
		Reverse	TCCCTCCATTTCGACTGTTC			
C-type lectin receptor a (*clra*)[a]	AY572832	Forward	CGAATCTTCAATCATGGAGAAG	0.997	101.1	117
		Reverse	TTCAGCCCCTGGGTATTTTG			
C-type lectin domain family 3 member a (*clec3a*)[a]	EL698766	Forward	CCAACCGTTACTGGAGCACT	0.999	99.8	174
		Reverse	GGCTCCCCTTAACCCAGATA			
C-type lectin domain family 4 member e-like (*clec4e*) [a]	EG928463	Forward	CCACCAATCACGCAACAT	0.996	104.1	115
		Reverse	TCACGCCTCTTCACTTCTCA			
chemokine receptor 1-like (*ccr1*) [a]	EG825373	Forward	GACCACGGGATGAACTTTGT	0.999	102.4	149
		Reverse	CACACCTCGGTTTCCACTTT			
C-X-C chemokine receptor type 1-like (*cxcr1*) [a]	CX355704	Forward	ATGCTGATTCCCCCTACTCC	0.994	99.6	103
		Reverse	ACACTGCTCAAGCCCAAGAT			
CC motif chemokine 13 (*ccl13*)	BT048088	Forward	ACTCCTCCTGGGACTGCTCT	0.986	99.1	109
		Reverse	CCTCTTTGGGTGGAACTTCA			
CC chemokine (*ccl*)	EG850594	Forward	TTCCCTGTGTCAATGCTGTC	0.998	96.6	137
		Reverse	GGTGGTGTTCTGTGTGTCCA			
hepcidin antimicrobial peptide (*hamp*)	BT125319	Forward	ATGAATCTGCCGATGCATTTC	0.994	93.5	134
		Reverse	AATGGCTTTAGTGCTGGCAG			
cathelicidin antimicrobial peptide (*camp*)	AY360357	Forward	AGACTGGCAACACCCTCAAC	0.990	104.9	112
		Reverse	TTGCCTCTTCTTGTCCGAAT			
cathepsin L1-like (*ctsl1*)	EG876363	Forward	GTTGACTGCTCCGGTGATTT	0.997	93.5	147
		Reverse	GTAGCGGCACTTCTTGTCCT			
lipase e, hormone-sensitive (*lipe*)	NM_001140535	Forward	ACCCAACTTTCCACGTCAAG	0.996	100.8	137
		Reverse	CAGTAGATCCCCGATGTCGT			
complement factor d (*cfd*)	BT058155	Forward	ATCCGCTCAGTGGTTCTTCA	0.998	96.6	147
		Reverse	TTGACCTCGGGACTGTAAGG			
cholesterol 25-hydroxylase-like protein a (*ch25ha*)	BT046542	Forward	TAGAGCTGTGATGCTAGTTTAC	0.990	102.9	106
		Reverse	ACCCAGTAGCACTGAGAAGTC			
cytokine-inducible sh2-containing protein (*cish*)	BT057484	Forward	TGGAGCCACGTCAGACATAA	0.997	104.3	153
		Reverse	GCACCATGTGTTTTCCAGTG			
e3 ubiquitin-protein ligase znrf1 (*znrf1*)	EG922586	Forward	CAGCACGTCATCGTTGTAGG	0.983	97.4	103
		Reverse	CAAGTGTCCTGTCTGCTCCA			
claudin-4, paralogue a (*cldn4-a*)[b]	BT125246	Forward	GGCCAGATGCAGTGTAAGGT	0.986	99.1	110
		Reverse	AAGATGCCGACGATACCTGT			
claudin-4, paralogue b (*cldn4-b*)[a, b]	BT048350	Forward	CAACATCGTGACTGCTCAGG	0.998	98.9	108
		Reverse	GGTAAGGCCAGAAGGGAGTC			
cyclin-y-like (*ccny*)	EG918014	Forward	CAACTTGAGCTTCCCTCTGG	0.996	93.7	111
		Reverse	TTCTTGCCAGCTCTCCTAGC			
Normalisers						
60S ribosomal protein 32 (*rpl32*)	BT043656	Forward	AGGCGGTTTAAGGGTCAGAT	0.997	105.5	119
		Reverse	TCGAGCTCCTTGATGTTGTG			
eukaryotic translation initiation factor 3 subunit D (*eif3d*)	GE777139	Forward	CTCCTCCTCCTCGTCCTCTT	0.998	103.8	105
		Reverse	GACCCCAACAAGCAAGTGAT			

Transcript names were taken from BLASTx searches against NCBI's non-redundant (nr) amino acid sequence using the nucleotide query sequences.
[a] These transcripts were selected from the only-RP identified list, and the rest of the transcripts were selected from the SAM- and RP-overlapping gene list.
[b] Each paralogue-specific primer (i.e. forward or reverse) was designed to contain at least 3 base mismatched sequence with the other paralogue.

and 110% were used for qPCR assays (Table 1).

Prior to the qPCR assays, in order to select endogenous controls (i.e. normaliser genes), the expression of seven candidate normalisers [i.e. *60S ribosomal protein 32* (*rpl32*), *elongation factor 1 alpha-1* (*ef1a1*), *elongation factor 1 alpha-2* (*ef1a2*), *polyadenylate-binding protein, cytoplasmic 1* (*pabpc1*), *eukaryotic translation initiation factor 3 subunit D* (*eif3d*), *ATP binding cassette sub-family f member 2* (*abcf2*), and *RNA polymerase 2* (*polr2*)] was measured in duplicate for 50% of the experimental samples (3 fish per treatment) as described below. Then, the resulting fluorescence cycle threshold (C_T) values were analysed by geNorm using the qBase software [45]. As suggested by geNorm, two normaliser transcripts, *eif3d* and *rpl32*, which showed low M-values (i.e. $M < 0.15$; a measure of transcript expression stability) and a comparable expression (i.e. C_T values) in all samples, were selected for the qPCR assays in the current study. Thereafter, the transcript (mRNA) levels of the GOIs were measured using cDNA template representing 5 ng of input RNA per PCR reaction. The GOIs and endogenous controls were tested in triplicate in all 23 samples using 384-well plates, including a no-template control. Using the ViiA 7 Software, Relative Quantification Study Application (Version 1.2.3) (Applied Biosystems), the relative quantity (RQ) of each tested transcript was calculated through normalisation to both endogenous control transcripts. Also, the amplification efficiencies of all genes' primer pairs were incorporated into the calculations, and the RQ value of each transcript was calibrated to a sample that showed the lowest normalised expression level (i.e. assigned an RQ value = 1).

The normality of data (i.e. RQ values) was tested using the Kolmogorov-Smirnov normality test. One-way ANOVA was used to determine the differences among groups. This analysis was followed by Tukey's multiple comparisons *post hoc* test to identify significant

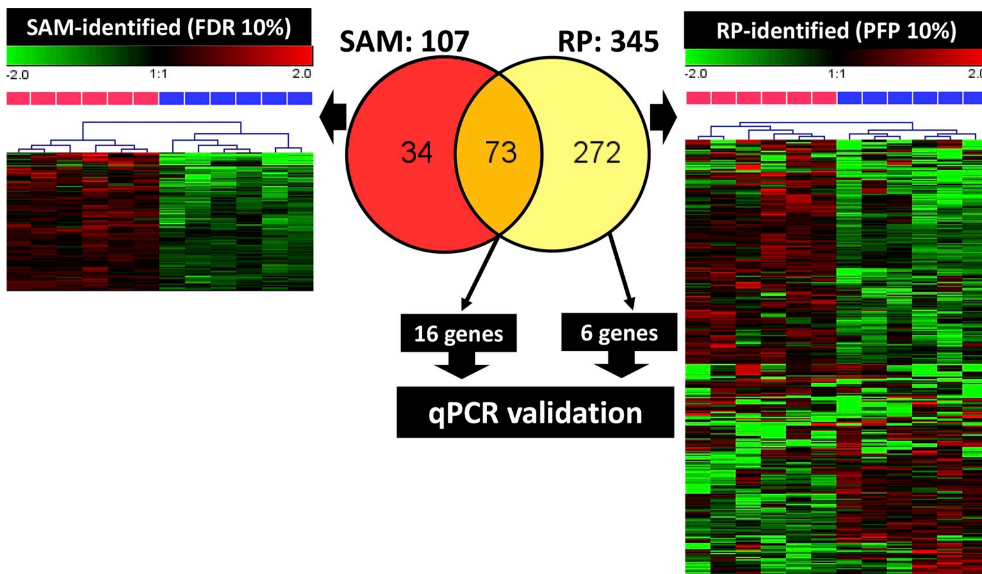

Fig. 1. Overview of microarray results. The *R. salmoninarum* bacterin-responsive probes identified by SAM (FDR < 0.1) and RP (PFP < 0.1) analyses (n = 6). The heat maps represent clustering of samples based on microarray data of the differentially expressed probes (DEP) identified by each analysis. Samples from the PBS control group are shown in cobalt boxes, whereas magenta boxes indicate samples from the High-Rs group. The full list of DEP by *R. salmoninarum* bacterin is shown in Supplemental Table S1.

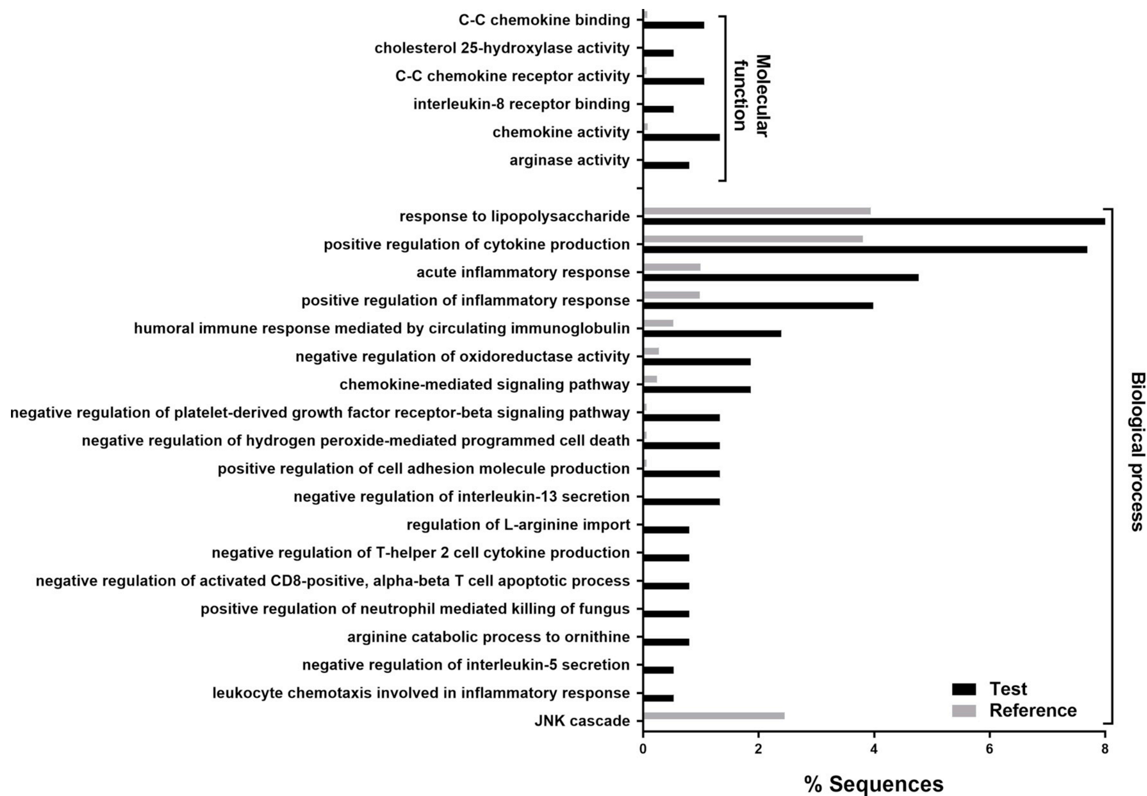

Fig. 2. Selected significantly enriched GO terms. A subset of significantly enriched GO terms associated with immune responses were selected; the full list of enriched GO terms is shown in Supplemental Table S2. The bar chart depicts the percentage of *R. salmoninarum* bacterin-responsive probes in Atlantic salmon head kidney [Test; SAM (FDR < 0.1) and/or RP (PFP < 0.1)] that were annotated with each GO term (Biological Process and Molecular Function), compared with the Reference (i.e. the entire 44K microarray).

differences ($p \leq 0.05$) between groups. These statistical analyses were performed using the Prism package v7.0 (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Microarray results

A 44K microarray platform was used to profile the response of Atlantic salmon head kidney to *R. salmoninarum* bacterin (Fig. 1). We used RP and SAM methods to identify Atlantic salmon transcripts differentially expressed in response to formalin-killed *R. salmoninarum* (i.e. High-Rs group vs. PBS control). SAM found 106 probes up-regulated in response to formalin-killed *R. salmoninarum* injection, but it only identified one down-regulated probe (i.e. *cyclin-y-like*) in response to bacterin injection. On the other hand, RP identified 345 DEP in response to formalin-killed *R. salmoninarum* injection in Atlantic salmon head kidney: 219 and 126 up- and down-regulated probes, respectively.

Fig. 3. Unsupervised clustering on pathway deregulation score (PDS) of microarray data of individuals (n = 6) from High-Rs and PBS groups. Samples from High-Rs and PBS control groups are shown in maroon and azure, respectively.

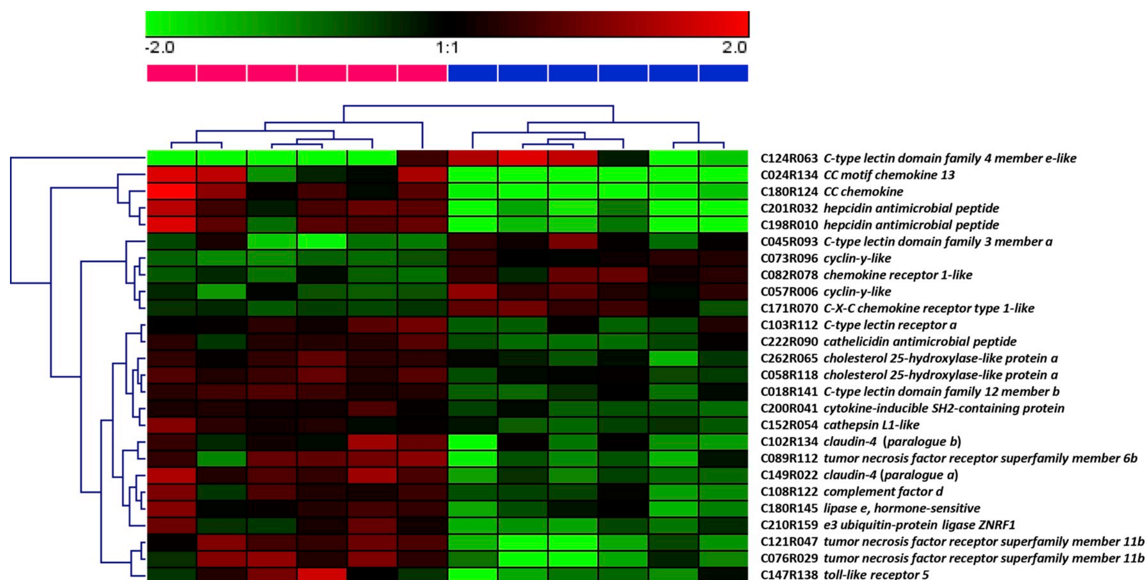

Fig. 4. Hierarchical clustering analyses of samples using microarray data of the differentially expressed probes (DEP) subjected to the qPCR validation. Samples from the PBS control group are shown in cobalt boxes, whereas magenta boxes indicate samples from the High-Rs group. Repeated gene names show different DEP that represent an identical transcript.

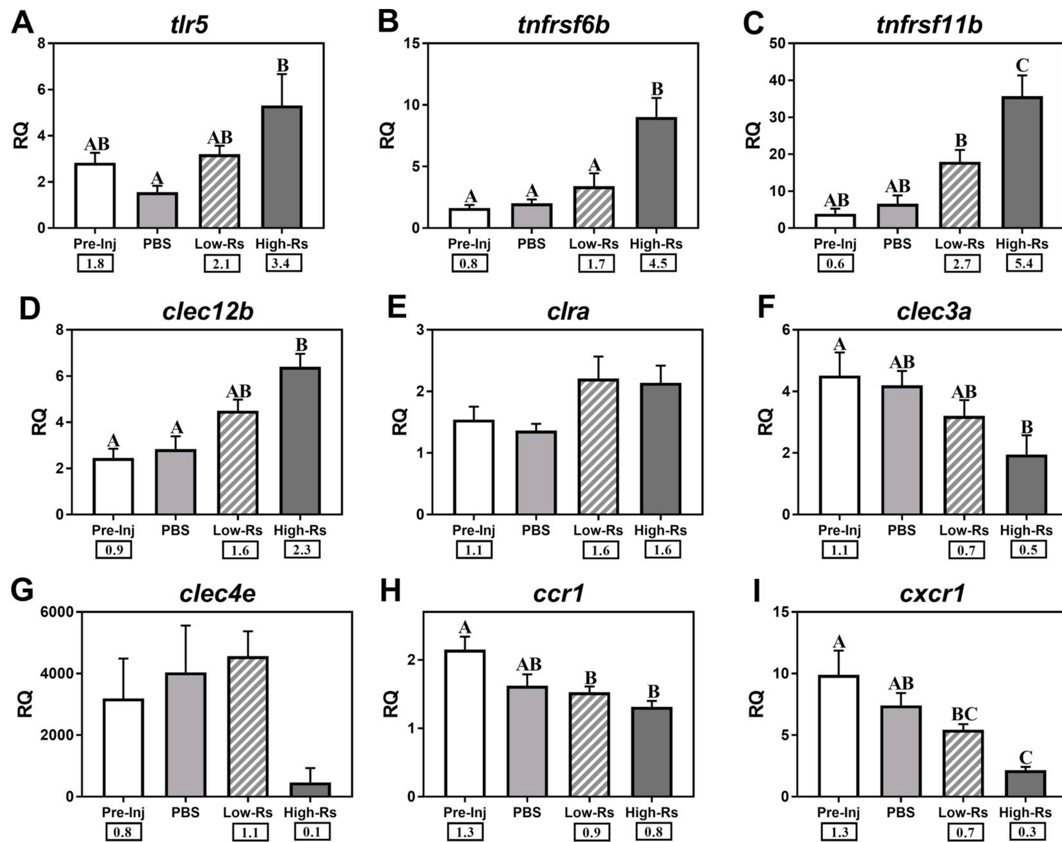

Fig. 5. qPCR for *R. salmoninarum* bacterin-responsive transcripts playing roles as PRRs or other immune receptors. Data are presented as mean ± SE. Different letters indicate the significant differences between the groups ($p < 0.05$). The fold-change values compared with the PBS group (e.g. High-Rs/PBS) are shown below the figures. Fish were sampled before injection (Pre-Inj; $n = 5$) or 24 h after injection with phosphate-buffered saline (PBS; $n = 6$), a low (Low-Rs; $n = 6$) or a high (High-Rs; $n = 6$) dose of formalin-killed *R. salmoninarum*.

There were 73 DEP overlapping between SAM- and RP-identified transcript lists, whereas 34 and 272 DEP were only found by SAM and RP, respectively (Fig. 1). Supplemental Table S1 provides additional details on the SAM and RP-identified probes in the current study.

As shown in Fig. 1, all of the samples in either PBS or High-Rs group clustered together based on the expression of SAM- and RP-identified genes. This suggests that the expression profiles of the transcripts in both SAM and RP lists are comparable in their ability to segregate individuals by treatment (High-Rs or PBS). Using Fisher's exact test (FDR cutoff of 5%), 79 GO terms (i.e. 72 over- and 7 under-represented GO terms), reduced to the most specific terms, were determined to be enriched or depleted in the global *R. salmoninarum* bacterin-responsive gene list (i.e. all probes identified by RP and/or SAM) compared to the whole microarray dataset (Supplemental Table S2). Fig. 2 shows a subset of selected enriched GO terms that are associated with immune responses. Molecular Function (MF) and Biological Process (BP) GO terms related to chemokine and cytokine regulation and production (e.g. chemokine-mediated signalling pathway, positive regulation of cytokine production and C–C chemokine receptor activity) were over-represented, whereas BP terms related to JNK cascade were under-represented in our gene list. Moreover, GO terms related to the anti-bacterial response, and bacterial elimination, were over-represented in the gene list identified as *R. salmoninarum* bacterin-responsive by RP and/or SAM in the present study.

3.2. Pathway analyses

Pathifier analysis found several pathways associated with immune responses to be dysregulated in response to *R. salmoninarum* bacterin injection (Fig. 3). Several identified dysregulated pathways (i.e. chemokine receptor binding and cytokine activity) were similar to the over-represented GO terms in *R. salmoninarum* bacterin-responsive gene list identified by Fisher's exact test. Moreover, Pathifier identified dysregulation in pathways associated with regulation of immune responses (e.g. positive regulation of interleukin 6 secretion, regulation of cytokine production involved in immune response and negative regulation of innate immune response; Fig. 3).

3.3. qPCR validation

Sixteen *R. salmoninarum* bacterin-responsive transcripts overlapping between SAM and RP gene lists, as well as 6 *R. salmoninarum* bacterin-responsive transcripts identified only by RP (i.e. 22 transcripts in total), were subjected to qPCR validation. Supplemental Table S1 shows the comparison between microarray and qPCR results (i.e. fold-changes of transcripts representing microarray-identified probes). Six *R. salmoninarum* bacterin-responsive transcripts identified only by RP were studied by qPCR (Table 1). Fig. 4 shows the clustering analysis of microarray results for the genes selected for qPCR validation. All qPCR-analysed genes showed the same fold-change direction to microarray results (Supplemental Table S1). The microarray results were significantly validated ($p \leq 0.05$) by qPCR assays for 15 of 16 identified genes overlapping between SAM and RP, and significant validation (i.e. High-Rs versus PBS control) was only not seen for *camp*. The microarray results for only 2 [i.e. *C-X-C chemokine receptor type 1-like* (*cxcr1*) and *claudin-4, paralogue b* (*cldn4-b*)] of 6 RP-only identified transcripts were significantly confirmed by qPCR, although the other 4 transcripts showed the same fold-change directions in both microarray and qPCR analyses.

The expression levels of 9 transcripts playing putative roles as

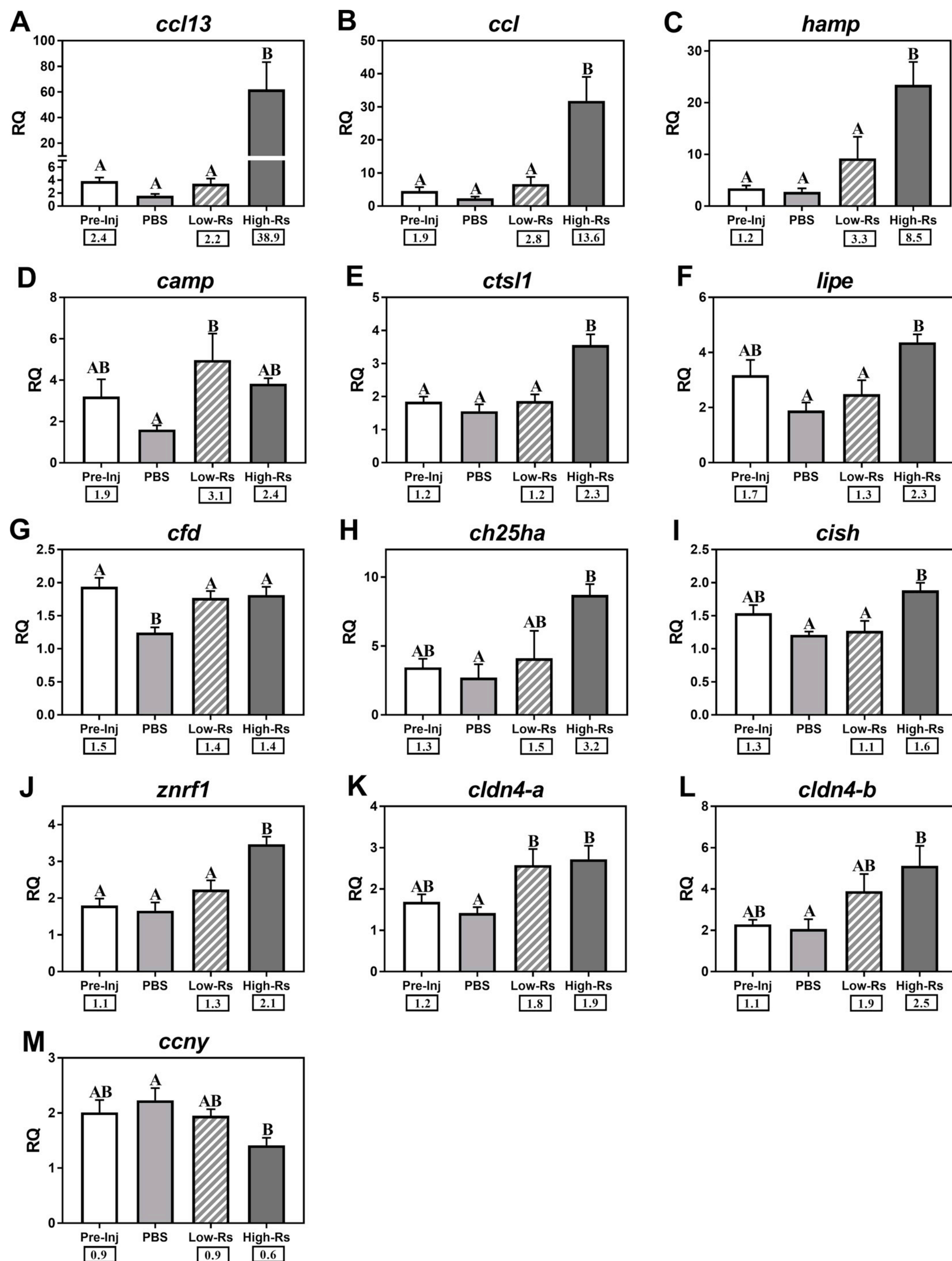

Fig. 6. qPCR for *R. salmoninarum* bacterin-responsive transcripts playing roles as immune effectors. Data are presented as mean ± SE. Different letters indicate the significant differences between the groups ($p < 0.05$). The fold-change values compared with the PBS group (e.g. High-Rs/PBS) are shown below the figures. Fish were sampled before injection (Pre-Inj; $n = 5$) or 24 h after injection with phosphate-buffered saline (PBS; $n = 6$), a low (Low-Rs; $n = 6$) or a high (High-Rs; $n = 6$) dose of formalin-killed *R. salmoninarum*.

pattern recognition receptors (PRRs) or other immune receptors were subjected to qPCR validation (Fig. 5). The transcript levels of *toll-like receptor 5* (*tlr5*), *tumor necrosis factor receptor superfamily member 6b* (*tnfrsf6b*) and *tumor necrosis factor receptor superfamily member 11b* (*tnfrsf11b*) were up-regulated in response to *R. salmoninarum* bacterin injection in the High-Rs group compared to the PBS group (3.4-, 4.5- and 5.4-fold increase, respectively; Fig. 5A–C). We also subjected 4 members of C-type lectin receptor family to qPCR assays. *C-type lectin domain family 12 member b* (*clec12b*) was identified by both SAM and RP in microarray analyses, whereas the rest [i.e. *C-type lectin receptor a* (*clra*), *C-type lectin domain family 3 member a* (*clec3a*) and *C-type lectin domain family 4 member e-like* (*clec4e*)] were selected from the RP-only identified gene list. The expression of *clec12b* significantly increased by 2.3-fold in the High-Rs compared with the PBS group, but no significant up-regulation was observed for *clra* (Fig. 5D–E). Also, the expression of *clec3a* and *clec4e* did not significantly vary between High-Rs and PBS, although they showed the same fold-change direction in qPCR and microarray results (Fig. 5F–G). Moreover, *clec3a* showed a significant

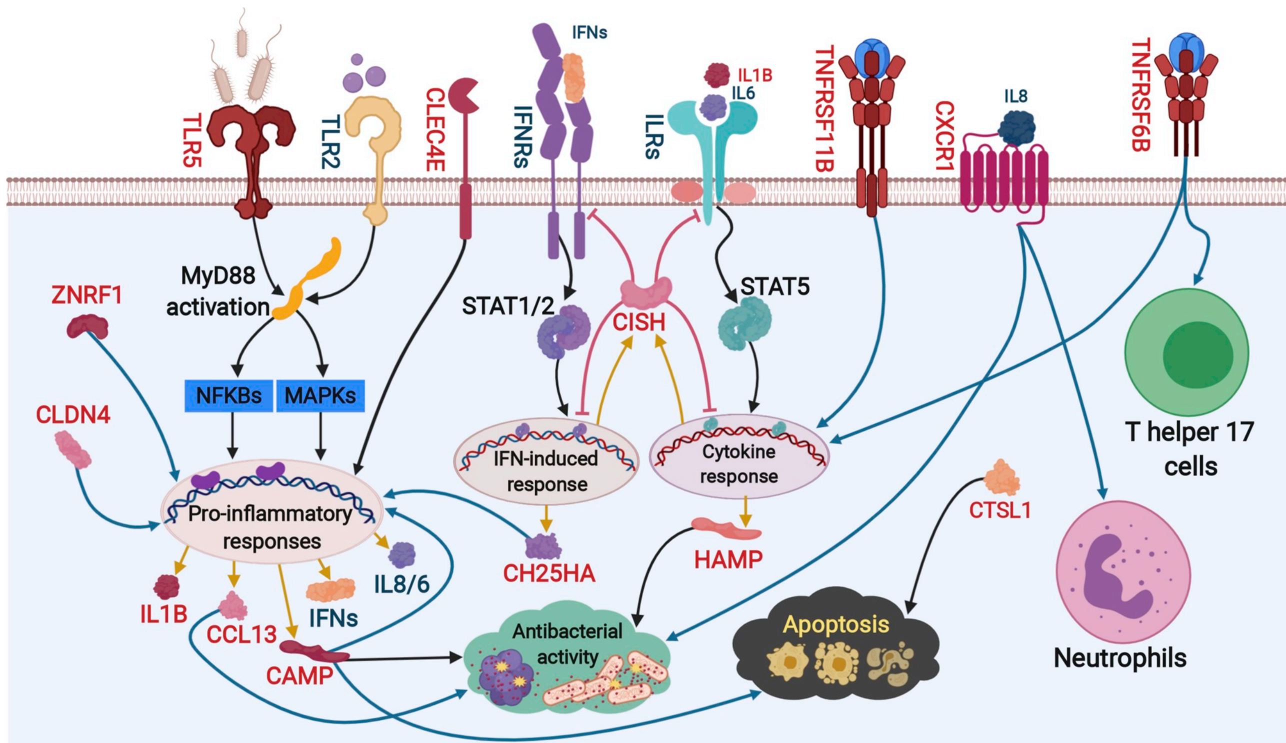

Fig. 7. The putative pathways differentially regulated by *R. salmoninarum*-derived antigens in Atlantic salmon head kidney. This figure was developed using the identified genes in this study and their known functions and regulatory pathways in mammals as explained in the discussion section [58,59,64,65,77,79,82,83,85–87]. The *R. salmoninarum* bacterin-responsive genes are shown in red font. The microarray results were validated by qPCR for all of the shown genes except for *camp* and *clec4e*. Black and blue arrows show activatory and regulatory effects, respectively. Yellow arrows show gene expression induction, whereas red lines indicate inhibitory effects. Oval circles and clouds reflect gene expression activation and biological processes, respectively. TLR (Toll-like receptor), MyD88 (myeloid differentiation primary response 88), NFKB (nuclear factor kappa-B), MAPK (mitogen-activated protein kinase), ILs (interleukins), CCL13 (CC motif chemokine 13), CAMP (cathelicidin antimicrobial peptide), ZNRF1 (e3 ubiquitin-protein ligase znrf1), CLDN4 (claudin-4), IFNs (interferons), IFNRs (IFN receptors), CLEC4E (C-type lectin domain family 4 member e-like), STAT (signal transducer and activator of transcription), CH25HA (cholesterol 25-hydroxylase-like protein a), CISH (cytokine-inducible sh2-containing protein), HAMP (hepcidin antimicrobial peptide), CTSL1 (cathepsin L1-like), TNFRSF11B (tumor necrosis factor receptor superfamily member 11b), TNFRSF6B (tumor necrosis factor receptor superfamily member 6b), CXCR1 (C-X-C chemokine receptor type 1-like).

down-regulation when comparing the High-Rs with Pre-Inj group. Two RP-only identified down-regulated chemokine receptors [i.e. *chemokine receptor 1-like* (*ccr1*) and *cxcr1*; Fig. 5H–I] had the same fold-change direction in both microarray and qPCR analyses, although significant differences between High-Rs and PBS groups were only seen for *cxcr1*. There appeared to be a dose-dependent response to *R. salmoninarum* bacterin for *cxcr1*.

The qPCR results of the 13 *R. salmoninarum* bacterin-responsive transcripts playing putative roles as immune effectors are shown in Fig. 6. All of these transcripts were microarray-identified as significantly *R. salmoninarum* bacterin-responsive by both SAM and RP, except for *cldn4-b*, which was selected from the RP-only identified gene list. The expression levels of *CC motif chemokine 13* (*ccl13*), *CC chemokine* (*ccl*) and *hamp* were strongly induced in High-Rs group (38.9-, 13.6- and 8.5-fold increase in comparison with PBS, respectively; Fig. 6A–C) compared to the other groups. Although *camp* showed the same fold-change direction to microarray results, significant up-regulation was only seen in Low-Rs group compared to the PBS group (Fig. 6D). There was more than 2-fold up-regulation in the levels of *cathepsin L1-like* (*ctsl1*) and *lipase e, hormone-sensitive* (*lipe*) in the High-Rs group compared with the PBS group (Fig. 6E–F). The transcript levels of *complement factor d* (*cfd*) were slightly (1.4-fold), but significantly, up-regulated by *R. salmoninarum* bacterin injection in both Low-Rs and High-Rs groups compared with the PBS group (Fig. 6G). The levels of *cholesterol 25-hydroxylase-like protein a* (*ch25ha*), *cytokine-inducible sh2-containing protein* (*cish*) and *e3 ubiquitin-protein ligase znrf1* (*znrf1*) were induced (3.2-, 1.6- and 2.1-fold, respectively; Fig. 6H–J) in High-Rs group compared to the PBS control. Both paralogues of *claudin-4* (76% similarity on the nucleotide level) were found to be responsive; however, significant induction of *cldn4-a* compared with PBS was seen in both Low-Rs and High-Rs groups, whereas *cldn4-b* levels were only significantly increased in the High-Rs group (Fig. 6K-L). In this study, there were two microarray-identified probes (C057R006: 0.47-fold, RP-identified probe; C073R096: 0.47-fold, RP- and SAM-identified probe) representing a *cyclin-y-like* (*ccny*) transcript. qPCR assays confirmed a 0.6-fold down-regulation for *ccny* in the High-Rs group compared to the PBS group (Fig. 6M). Among all the genes assessed by qPCR in the current study, only *camp*, *cfd* and *cldn4-a* showed a significant difference between Low-Rs and PBS groups (Fig. 6D, G, K).

4. Discussion

The present study was conducted to develop a better understanding of the molecular mechanisms underlying the Atlantic salmon head kidney response to *R. salmoninarum* bacterin. We used SAM and RP, which have different statistical algorithms [35,46], to determine the differentially expressed transcripts upon *R. salmoninarum* bacterin injection. SAM and RP identified 107 and 345 DEP, respectively, in head kidney of Atlantic salmon injected with *R. salmoninarum* bacterin. Additionally, the majority of *R. salmoninarum* bacterin-responsive transcripts identified herein did not show a robust gene dysregulation. For example, only 5 of the DEP were up-regulated greater than 5-fold by *R. salmoninarum* bacterin. Other than the current study, there is no published data on the transcriptome responses of Atlantic salmon to *R. salmoninarum* or its bacterin. Using SSH, a previous study identified 132 ESTs responsive to *R. salmoninarum* infection in Chinook salmon [16].

However, previous studies profiling the transcriptome responses of fishes to different pathogen-associated molecular patterns (PAMPs) identified larger numbers of responsive genes compared to the current study. For example, an SSH-based study identified 1655 and 1239 ESTs in the spleen and head kidney, respectively, of Atlantic cod (*Gadus morhua*) injected with formalin-killed *A. salmonicida* (i.e. a Gram-negative pathogen) [47]. Our previous microarray study identified more than 3000 DEP in Atlantic salmon macrophage-like cells stimulated with virus-like dsRNA PAMP [i.e. polyriboinosinic polyribocytidylic acid (pIC)] [28]. The differences observed between the current and previous studies may be caused by PAMP-specific responses that are activated by various PRRs. For example, virus-like dsRNA activates signalling pathway downstream of TLR3 [48]. TLR4 recognises the lipopolysaccharides (LPS) of Gram-negative bacteria, whereas the peptidoglycan of Gram-positive bacteria is detected by TLR2 [49]. Despite some similarities, human exhibits distinct responses and immunoregulatory patterns to Gram-positive and Gram-negative bacteria [50,51]. It has been noted that Gram-negative bacteremia causes stronger cytokine induction compared to Gram-positive bacteremia in human [52]. Likewise, the numbers of differentially expressed genes (i.e. more than 2-fold down- or up-regulation) in the kidney of Japanese flounder (*Paralichthys olivaceus*) injected with formalin-killed Gram-negative bacterial cells (*Edwardsiella tarda*) at 6 and 12 HPI were greater than those with formalin-killed Gram-positive bacterial cells (*Streptococcus iniae*) [53]. The lower number of DEP by *R. salmoninarum* bacterin observed herein compared to other bacterins (e.g. formalin-killed *A. salmonicida*) or PAMPs (e.g. virus-like dsRNA) in previous studies may be related to the immune pathways activated differently in response to various immunogens (e.g. Gram-positive and Gram-negative bacteria) as well as species-specific immune responses and experimental design.

To validate the microarray results, a subset of *R. salmoninarum* bacterin-responsive transcripts was subjected to qPCR validation. Since RP and SAM use different approaches to identify significant responsive genes [33,35], the *R. salmoninarum* bacterin-responsive transcripts overlapping between these two statistical methods were considered as the most trusted transcript list, and most of the selected transcripts for qPCR assays were taken from this list. All qPCR-assessed transcripts showed the same fold-change direction as the microarray results, although microarray results were not significantly validated for 5 (i.e. 1 SAM- and RP-identified and 4 RP-only-identified transcripts) out of 22 transcripts. The differences between qPCR and microarray results may be explained by the variations in both stringency level and statistical methods used for data analyses. Among all the qPCR-assessed transcripts, *R. salmoninarum* bacterin induction in Low-Rs group was only observed for *cfd, cldn4-a* and *camp*, and it reflects a dose-dependent response of Atlantic salmon to *R. salmoninarum* bacterin.

Fig. 7 shows the putative pathways activated by *R. salmoninarum* bacterin in Atlantic salmon. The levels of *tlr5*, *tnfrsf6b* and *tnfrsf11b* transcripts were up-regulated in the High-Rs group compared to the PBS control. Mammalian TLR5 is a PRR recognising bacterial flagellin, resulting in activation of MyD88-dependent pathway and the production of pro-inflammatory cytokines [54]. Flagellin recognition of TLR5 and TLR5-mediated NFKB activation have been reported in teleosts (e.g. rainbow trout and Japanese flounder) [55]. Also, *tlr5* was shown to be up-regulated in the kidney of striped catfish (*Pangasianodon hypophthalmus*) in response to *E. tarda* (i.e. a motile, Gram-negative bacterium) at 24 HPI [56]. Unlike *E. tarda, R. salmoninarum* is a non-motile species [6]. Therefore, it is not clear if the induction of Atlantic salmon *tlr5* by *R. salmoninarum* bacterin seen herein occurred following the TLR5-dependent pathogen recognition or activation of immune responses by other PRRs. Although a tissue-dependent response of *tlr5* to *S. iniae* (a Gram-positive, non-motile bacterium) was observed in turbot (*Scophthalmus maximus*) [57], further studies are needed to determine the function of teleost TLR5 in response to bacteria lacking flagella.

Mammalian TNFRSF6B (alias DcR3) is involved in regulation of Th17 activity and cytokine immune responses [58] (Fig. 7). In addition to its function in osteoclastogenesis, TNFRSF11B (alias Osteoprotegerin) can regulate LPS-induced cytokine responses of mice [59]. We found that *R. salmoninarum* bacterin stimulation influenced pathways related to the regulation of cytokine production, and this may be related to the regulatory role of *tnfrsf11b* in cytokine responses. Although up-regulation of *tnfrsf6b* and *tnfrsf11b* in response to viral and bacterial PAMPs was seen in Atlantic salmon mononuclear phagocytes [60], the function of these transcripts in Atlantic salmon immune response remained unknown. A previous *in vitro* study reported up-regulation of *tnfa* in *R. salmoninarum*-infected rainbow trout macrophages at 24 HPS [14], but *tnf* induction was not seen in the present study. This may be explained by differences in response to killed and live *R. salmoninarum* or species-specific responses as well as *in vivo* versus *in vitro* studies.

In the present study, transcripts encoding different members of the C-type lectin domain family variably (e.g. up-regulation of *clec12b* and down-regulation of *clec3a*) responded to *R. salmoninarum* bacterin in Atlantic salmon. C-type lectin receptors can activate (e.g. as PRR) a wide variety of immune signalling pathways and are involved in a large number of immune processes (e.g. antibacterial, antifungal and cell death) [61]. *clec12b* is a poorly characterised transcript and does not have any known ligand [62]. *clec3a* was found to be associated with mammalian tumor progression [63]. As in the microarray experiment, qPCR showed *clec4e* to be down-regulated in response to *R. salmoninarum* bacterin; however, this change was not statistically significant in the qPCR analysis. Mammalian *clec4e* (alias *mincle*) was described to trigger the inflammatory responses by recognising pathogenic fungi or yeast [64]. However, the function of these genes in antibacterial responses of teleost fish is yet to be determined.

Microarray analyses in the present study revealed suppression of Atlantic salmon chemokine receptors (*ccr1* and *cxcr1*) in response to *R. salmoninarum* bacterin, although these results were only significantly validated for *cxcr1* (alias *il8ra*). A previous study showed the up-regulation of other chemokine receptors (i.e. *cxcr4* and *ccr7*) in rainbow trout macrophages following infection with *R. salmoninarum* for 24 h [14]. This suggests that various cytokine receptors may have different regulation (e.g. tissue- or species-specific) and function in salmonid response to *R. salmoninarum*. Mammalian CXCR1 is a specific receptor for IL8 (Fig. 7), which recruits neutrophils to the inflammation site and enhances the antibacterial activity of neutrophils [65]. As in the current findings, *cxcr1* was down-regulated in peripheral blood leucocytes of fugu (*Takifugu rubripes*) 12 h after LPS stimulation [66]. Therefore, the suppressed expression of *cxcr1* seen herein may be attributed to the immunoregulative responses caused by inflammation, since we observed opposite regulation of chemokines (*ccl13* and *ccl*) and *cxcr1* in response to *R. salmoninarum* bacterin stimulation. Our qPCR results, alongside over-representation of molecular functions related to chemokine receptor activity and dysregulation of pathways associated with chemokine receptor and binding, reflect the importance of chemokines in responses of Atlantic salmon to *R. salmoninarum* bacterin (Fig. 7).

Thirteen *R. salmoninarum* bacterin-responsive transcripts with putative roles as immune effectors were subjected to qPCR validation. All of these transcripts, except for *ccny*, were up-regulated in response to *R. salmoninarum* bacterin injection, and the microarray results were confirmed for all of them except for *camp*. Fig. 7 depicts the putative pathways dysregulated in response to *R. salmoninarum* bacterin in Atlantic salmon. In agreement with the gene expression responses of *ccl13* and *ccl*, pathways associated with cytokine and chemokine activity were found to be dysregulated in *R. salmoninarum* bacterin-injected Atlantic salmon (Fig. 7). *ccl* studied herein is not a fully characterised transcript, and further structural studies on this transcript are needed to have a better understanding of its function. Human CCL13 exhibits antibacterial activity against *Pseudomonas aeruginosa* [67]. Although *ccl13* showed induction in pIC-stimulated Atlantic salmon and cod [28,68], further studies are needed to elucidate its antibacterial role in teleosts.

As in the current study, *hamp* and *camp* were up-regulated in Atlantic salmon and Atlantic cod head kidney 24 h after injection of live *A. salmonicida* vaccine [21] and formalin-killed *A. salmonicida* [47], respectively. Synthetic or recombinant HAMP of teleost fishes (i.e. rainbow trout, medaka (*Oryzias melastigma*) and European seabass (*Dicentrarchus labrax*)] showed bactericidal activity against both Gram-positive and Gram-negative bacteria [69–71]. Using a zebrafish model, it has been shown that the regulatory role of HAMP in iron metabolism positively contributes to its antimicrobial activity [72]. The anti-bacterial activity of rainbow trout CAMP against both Gram-positive and Gram-negative pathogens is mediated through membrane permeabilisation [73] (Fig. 7). Besides these AMPs, the transcript level of *cfd* was slightly up-regulated in both Low-Rs and High-Rs groups compared to PBS control. Mammalian CFD is involved in the alternative complement pathway [74]. While the current study showed the induction of *cfd*, *hamp* and *camp* by *R. salmoninarum* bacterin, the bactericidal functions of these genes against *R. salmoninarum* remain undetermined, and warrant further investigation.

We identified two *R. salmoninarum* bacterin-responsive up-regulated transcripts (i.e. *ch25ha* and *lipe*) involved in lipid metabolism that may also play roles in immune responses. Lipe (Lipase E, hormone sensitive) contributes to triacylglycerol hydrolysis in mammals [75]. Although insect Lipase exhibits antibacterial activity against Gram-positive and Gram-negative bacteria [76], the potential immune-related function of Lipases in teleosts or mammals remains unknown. Besides cholesterol biosynthesis regulation, mammalian CH25H also exhibits antiviral activities, and both positively and negatively regulates the inflammatory responses following the production of bacterial or viral-induced IFNs (Fig. 7) [77]. A conserved antiviral function was found for teleost CH25H [spotted grouper (*Epinephelus coioides*)] [78], but its immunoregulatory role is not fully understood in teleost species.

We identified transcripts that play putative roles in regulation of inflammatory or immune responses. In the present study, *cish* and *znrf1* were found to be up-regulated in response to *R. salmoninarum* bacterin. Parallel to the putative regulatory function of CH25H, CISH suppresses the immune response through binding to phosphorylated residues of the cytokine receptors [79]. Rainbow trout *cish* transcripts were found to be up-regulated in cytokine-exposed leukocytes and head kidney of fish challenged with *Yersinia ruckeri* for 24 h [80], but the immunoinhibitory activity of salmonid CISH has not yet been functionally validated. Moreover, as shown in Fig. 7, mammalian ZNRF1 is a regulator of LPS-induced inflammatory responses [81]. Induction of *znrf1* in response to a high dose of *R. salmoninarum* bacterin in the current study suggests that this transcript may have some conserved roles in Atlantic salmon. In the present study, two paralogues of *cldn4* were up-regulated in response to *R. salmoninarum* bacterin. Although *clbn4-b* was only significantly up-regulated in the High-Rs group compared to the PBS control, *cldn4-a* induction occurred independent of *R. salmoninarum* bacterin dose, suggesting a paralogue-specific response of *cldn4* to a low dose of bacterin. Mice *clbn4* is associated with the attenuation of the induced inflammatory responses [82] (Fig. 7), but its role in immune responses of teleosts remains undetermined. Cathepsins are apoptotic regulators that accelerate cell death through degradation of anti-apoptotic proteins [83]. Accordingly, the *ctsl1* induction in the current study suggested that *R. salmoninarum* bacterin injection increases the pro-apoptosis process in Atlantic salmon head kidney.

We observed a down-regulation in *ccny* in response to *R. salmoninarum* bacterin; this gene is a member of a family of kinases involved in the regulation of various biological processes (e.g. cell progression, cancer and neuronal differentiation) in human [84]. Future structural and functional studies can provide a better understanding of the molecular functions of *ccny* in Atlantic salmon.

5. Conclusions

This was the first report on the transcriptome profile of Atlantic salmon head kidney response to *R. salmoninarum* bacterin, and it provided a broader picture of molecular processes underlying early Atlantic salmon responses to formalin-killed *R. salmoninarum* bacterin. Our results showed the activation of pathways associated with chemokine receptor, cytokine activity and regulation of immune responses. qPCR results showed that Atlantic salmon responds to *R. salmoninarum* bacterin in a dose-dependent manner. *R. salmoninarum* bacterin does not cause an extensive and strong gene regulation response in Atlantic salmon, and this may be related to the immunosuppressive functions of this pathogen. *R. salmoninarum* bacterin influenced the expression of transcripts with putative functions involving pathogen recognition, immune signalling, antimicrobial activity, cell death and regulation of immune responses. The differentially expressed genes identified herein can be used as biomarkers for future studies such as evaluating the influence of diets and feed ingredients on Atlantic salmon response to *R. salmoninarum*-derived antigens as well as the response of Atlantic salmon to BKD. Future genomics-based studies using *R. salmoninarum* pathogen are needed to expand our knowledge on molecular processes involved in Atlantic salmon response to BKD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.11.057.

References

[1] FAO, The State of World Fisheries and Aquaculture (SOFIA) FAO2016.
[2] FAO, The State of World Fisheries and Aquaculture (SOFIA), Yearbook FAO2017.
[3] FAO, FAO Year Book, Fishery and Aquaculture Statistics, FAO, Rome, 2010.
[4] A.E. Toranzo, B. Magarinos, J.L. Romalde, A review of the main bacterial fish diseases in mariculture systems, Aquaculture 246 (1–4) (2005) 37–61.
[5] K.D. Lafferty, C.D. Harvell, J.M. Conrad, C.S. Friedman, M.L. Kent, A.M. Kuris, E.N. Powell, D. Rondeau, S.M. Saksida, Infectious diseases affect marine fisheries and aquaculture economics, Annu. Rev. Mar. Sci. 7 (2015) 471–496.
[6] B. Austin, D.A. Austin, Aerobic gram-positive rods and cocci, in: B. Austin, D.A. Austin (Eds.), Bacterial Fish Pathogens, Springer, Cham, 2016, pp. 83–160.
[7] J. Fryer, J. Sanders, Bacterial kidney disease of salmonid fish, Annu. Rev. Microbiol. 35 (1) (1981) 273–298.
[8] A. Evenden, T. Grayson, M. Gilpin, C. Munn, *Renibacterium salmoninarum* and bacterial kidney disease—the unfinished jigsaw, Annu. Rev. Fish Dis. 3 (1993) 87–104.
[9] D.G. Elliott, G.D. Wiens, K.L. Hammell, L.D. Rhodes, Vaccination against bacterial kidney disease, Fish Vaccination (2014) 255–272.
[10] Å. Fredriksen, C. Endresen, H. Wergeland, Immunosuppressive effect of a low molecular weight surface protein from *Renibacterium salmoninarumon* lymphocytes from Atlantic salmon (*Salmo salar* L.), Fish Shellfish Immunol. 7 (4) (1997) 273–282.
[11] J. Daly, S. Griffiths, A. Kew, A. Moore, G. Olivier, Characterization of attenuated *Renibacterium salmoninarum* strains and their use as live vaccines, Dis. Aquat. Org. 44 (2) (2001) 121–126.
[12] E. Jansson, O. Ljungberg, Detection of humoral antibodies to *Renibacterium salmoninarum* in rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* challenged by immersion and in naturally infected populations, Dis. Aquat. Org. 33 (2) (1998) 93–99.
[13] J.J. Campos-Perez, M. Ward, P.S. Grabowski, A.E. Ellis, C.J. Secombes, The gills are an important site of iNOS expression in rainbow trout *Oncorhynchus mykiss* after challenge with the Gram-positive pathogen *Renibacterium salmoninarum*, Immunology 99 (1) (2000) 153–161.

[14] T.H. Grayson, L.F. Cooper, A.B. Wrathmell, J.R. Evenden, J. Andrew, M.L. Gilpin, Host responses to *Renibacterium salmoninarum* and specific components of the pathogen reveal the mechanisms of immune suppression and activation, Immunology 106 (2) (2002) 273–283.

[15] J. Bethke, E. Arias-Muñoz, A. Yáñez, R. Avendaño-Herrera, *Renibacterium salmoninarum* iron-acquisition mechanisms and ASK cell line infection: virulence and immune response, J. Fish Dis. 42 (9) (2019) 1283–1291.

[16] L.D. Rhodes, S. Wallis, S.E. Demlow, Genes associated with an effective host response by Chinook salmon to *Renibacterium salmoninarum*, Dev. Comp. Immunol. 33 (2) (2009) 176–186.

[17] D.C. Metzger, D.G. Elliott, A. Wargo, L.K. Park, M.K. Purcell, Pathological and immunological responses associated with differential survival of Chinook salmon following *Renibacterium salmoninarum* challenge, Dis. Aquat. Org. 90 (1) (2010) 31–41.

[18] G. Hyatt, R. Melamed, R. Park, R. Seguritan, C. Laplace, L. Poirot, S. Zucchelli, R. Obst, M. Matos, E. Venanzi, Gene expression microarrays: glimpses of the immunological genome, Nat. Immunol. 7 (7) (2006) 686.

[19] M.L. Rise, S.R. Jones, G.D. Brown, K.R. von Schalburg, W.S. Davidson, B.F. Koop, Microarray analyses identify molecular biomarkers of Atlantic salmon macrophage and hematopoietic kidney response to *Piscirickettsia salmonis* infection, Physiol. Genom. 20 (1) (2004) 21–35.

[20] K.V. Ewart, J.C. Belanger, J. Williams, T. Karakach, S. Penny, S.C. Tsoi, R.C. Richards, S.E. Douglas, Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology, Dev. Comp. Immunol. 29 (4) (2005) 333–347.

[21] S.A.M. Martin, S.C. Blaney, D. Houlihan, C.J. Secombes, Transcriptome response following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*), Mol. Immunol. 43 (11) (2006) 1900–1911.

[22] A.R. Bridle, B.F. Koop, B.F. Nowak, Identification of surrogates of protection against yersiniosis in immersion vaccinated Atlantic salmon, PLoS One 7 (7) (2012) e40841.

[23] H. Lund, A.F. Bakke, I. Sommerset, S. Afanasyev, G. Schriwer, A. Thorisdottir, P. Boysen, A. Krasnov, A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon, Mol. Immunol. 106 (2019) 99–107.

[24] S.G. Jantzen, D.S. Sanderson, K.R. von Schalburg, M. Yasuike, F. Marass, B.F. Koop, A 44K microarray dataset of the changing transcriptome in developing Atlantic salmon (*Salmo salar* L.), BMC Res. Notes 4 (1) (2011) 88.

[25] A. Müller, B.J. Sutherland, B.F. Koop, S.C. Johnson, K.A. Garver, Infectious hematopoietic necrosis virus (IHNV) persistence in Sockeye Salmon: influence on brain transcriptome and subsequent response to the viral mimic poly (I:C), BMC Genomics 16 (1) (2015) 634.

[26] B.J. Sutherland, K.C. Hanson, J.R. Jantzen, B.F. Koop, C.T. Smith, Divergent immunity and energetic programs in the gills of migratory and resident *Oncorhynchus mykiss*, Mol. Ecol. 23 (8) (2014) 1952–1964.

[27] B.J. Sutherland, K.W. Koczka, M. Yasuike, S.G. Jantzen, R. Yazawa, B.F. Koop, S.R. Jones, Comparative transcriptomics of Atlantic *Salmo salar*, chum *Oncorhynchus keta* and pink salmon *O. gorbuscha* during infections with salmon lice *Lepeophtheirus salmonis*, BMC Genomics 15 (1) (2014) 200.

[28] K. Eslamloo, X. Xue, J.R. Hall, N.C. Smith, A. Caballero-Solares, C.C. Parrish, R.G. Taylor, M.L. Rise, Transcriptome profiling of antiviral immune and dietary fatty acid dependent responses of Atlantic salmon macrophage-like cells, BMC Genomics 18 (1) (2017) 706.

[29] S. Lien, B.F. Koop, S.R. Sandve, J.R. Miller, M.P. Kent, T. Nome, T.R. Hvidsten, J.S. Leong, D.R. Minkley, A. Zimin, F. Grammes, H. Grove, A. Gjuvsland, B. Walenz, R.A. Hermansen, K. von Schalburg, E.B. Rondeau, A. Di Genova, J.K.A. Samy, J. Olav Vik, M.D. Vigeland, L. Caler, U. Grimholt, S. Jentoft, D. Inge Våge, P. de Jong, T. Moen, M. Baranski, Y. Palti, D.R. Smith, J.A. Yorke, A.J. Nederbragt, A. Tooming-Klunderud, K.S. Jakobsen, X. Jiang, D. Fan, Y. Hu, D.A. Liberles, R. Vidal, P. Iturra, S.J.M. Jones, I. Jonassen, A. Maass, S.W. Omholt, W.S. Davidson, The Atlantic salmon genome provides insights into rediploidization, Nature 533 (2016) 200.

[30] T. Evelyn, An improved growth medium for the kidney disease bacterium and some notes on using the medium, Bull. Off. Int. Epizoot. 87 (1977) 511–513.

[31] A. Brazma, P. Hingamp, J. Quackenbush, G. Sherlock, P. Spellman, C. Stoeckert, J. Aach, W. Ansorge, C.A. Ball, H.C. Causton, Minimum information about a microarray experiment (MIAME)—toward standards for microarray data, Nat. Genet. 29 (4) (2001) 365.

[32] X. Xue, S.M. Hixson, T.S. Hori, M. Booman, C.C. Parrish, D.M. Anderson, M.L. Rise, Atlantic salmon (*Salmo salar*) liver transcriptome response to diets containing *Camelina sativa* products, Comp. Biochem. Physiol. Part D: Genom. Proteom. 14 (2015) 1–15.

[33] V.G. Tusher, R. Tibshirani, G. Chu, Significance analysis of microarrays applied to the ionizing radiation response, Proc. Natl. Acad. Sci. U.S.A. 98 (9) (2001) 5116–5121.

[34] T.D. Brown, T.S. Hori, X. Xue, C.L. Ye, D.M. Anderson, M.L. Rise, Functional genomic analysis of the impact of camelina (*Camelina sativa*) meal on Atlantic salmon (*Salmo salar*) distal intestine gene expression and physiology, Mar. Biotechnol. (2016) 1–18.

[35] F. Hong, R. Breitling, C.W. McEntee, B.S. Wittner, J.L. Nemhauser, J. Chory, RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis, Bioinformatics 22 (22) (2006) 2825–2827.

[36] S. Götz, J.M. García-Gómez, J. Terol, T.D. Williams, S.H. Nagaraj, M.J. Nueda, M. Robles, M. Talón, J. Dopazo, A. Conesa, High-throughput functional annotation and data mining with the Blast2GO suite, Nucleic Acids Res. 36 (10) (2008) 3420–3435.

[37] A. Conesa, S. Götz, J.M. García-Gómez, J. Terol, M. Talón, M. Robles, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21 (18) (2005) 3674–3676.

[38] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (1990) 403–410.

[39] A. Sturn, J. Quackenbush, Z. Trajanoski, Genesis: cluster analysis of microarray data, Bioinformatics 18 (1) (2002) 207–208.

[40] Y. Drier, M. Sheffer, E. Domany, Pathway-based personalized analysis of cancer, Proc. Natl. Acad. Sci. U.S.A. 110 (16) (2013) 6388–6393.

[41] A. Liberzon, A. Subramanian, R. Pinchback, H. Thorvaldsdóttir, P. Tamayo, J.P. Mesirov, Molecular signatures database (MSigDB) 3.0, Bioinformatics 27 (12) (2011) 1739–1740.

[42] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (4) (2009) 611–622.

[43] K. Eslamloo, A. Ghorbani, X. Xue, S.M. Inkpen, M. Larijani, M.L. Rise, Characterisation and transcript expression analyses of Atlantic cod *viperin*, Front. Immunol. 10 (2019) 311.

[44] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT–PCR, Nucleic Acids Res. 29 (9) (2001) 2002–2007.

[45] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (7) (2002) research0034. 1.

[46] G. Chu, J. Li, B. Narasimhan, R. Tibshirani, V. Tusher, SAM "significance Analysis of Microarrays" Users Guide and Technical Document, Stanford University, 2001.

[47] C.Y. Feng, S.C. Johnson, T.S. Hori, M. Rise, J.R. Hall, A.K. Gamperl, S. Hubert, J. Kimball, S. Bowman, M.L. Rise, Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*, Physiol. Genom. 37 (3) (2009) 149–163.

[48] L.A. O'neill, D. Golenbock, A.G. Bowie, The history of Toll-like receptors—redefining innate immunity, Nat. Rev. Immunol. 13 (6) (2013) 453.

[49] S. Mukherjee, S. Karmakar, S.P.S. Babu, TLR2 and TLR4 mediated host immune responses in major infectious diseases: a review, Braz. J. Infect. Dis. 20 (2) (2016) 193–204.

[50] R.J. Feezor, C. Oberholzer, H.V. Baker, D. Novick, M. Rubinstein, L.L. Moldawer, J. Pribble, S. Souza, C.A. Dinarello, W. Ertel, Molecular characterization of the acute inflammatory response to infections with Gram-negative versus Gram-positive bacteria, Infect. Immun. 71 (10) (2003) 5803–5813.

[51] K. Tietze, A. Dalpke, S. Morath, R. Mutters, K. Heeg, C. Nonnenmacher, Differences in innate immune responses upon stimulation with Gram-positive and Gram-negative bacteria, J. Periodontal. Res. 41 (5) (2006) 447–454.

[52] R. Abe, S. Oda, T. Sadahiro, M. Nakamura, Y. Hirayama, Y. Tateishi, K. Shinozaki, H. Hirasawa, Gram-negative bacteremia induces greater magnitude of inflammatory response than Gram-positive bacteremia, Crit. Care 14 (2) (2010) R27.

[53] H. Kondo, Y. Kawana, Y. Suzuki, I. Hirono, Comprehensive gene expression profiling in Japanese flounder kidney after injection with two different formalin-killed pathogenic bacteria, Fish Shellfish Immunol. 41 (2) (2014) 437–440.

[54] E.M.Y. Moresco, D. LaVine, B. Beutler, Toll-like receptors, Curr. Biol. 21 (13) (2011) R488–R493.

[55] J. Zhang, X. Kong, C. Zhou, L. Li, G. Nie, X. Li, Toll-like receptor recognition of bacteria in fish: ligand specificity and signal pathways, Fish Shellfish Immunol. 41 (2) (2014) 380–388.

[56] P.K. Jayaramu, G. Tripathi, A. Pavan Kumar, J. Keezhedath, M.K. Pathan, P.P. Kurcheti, Studies on expression pattern of toll-like receptor 5 (TLR5) in *Edwardsiella tarda* infected *Pangasianodon hypophthalmus*, Fish Shellfish Immunol. 63 (2017) 68–73.

[57] F. Liu, B. Su, Q. Fu, M. Shang, C. Gao, F. Tan, C. Li, Identification, characterization and expression analysis of TLR5 in the mucosal tissues of turbot (*Scophthalmus maximus* L.) following bacterial challenge, Fish Shellfish Immunol. 68 (2017) 272–279.

[58] A.M. Mueller, X. Pedré, S. Killian, M. David, A. Steinbrecher, The Decoy Receptor 3 (DcR3, TNFRSF6B) suppresses Th17 immune responses and is abundant in human cerebrospinal fluid, J. Neuroimmunol. 209 (1–2) (2009) 57–64.

[59] K. Maruyama, Y. Takada, N. Ray, Y. Kishimoto, J.M. Penninger, H. Yasuda, K. Matsuo, Receptor activator of NF-κB ligand and osteoprotegerin regulate proinflammatory cytokine production in mice, J. Immunol. 177 (6) (2006) 3799–3805.

[60] D.B. Iliev, L. Lagos, H.L. Thim, S.M. Jørgensen, A. Krasnov, J.B. Jørgensen, CpGs induce differentiation of Atlantic salmon mononuclear phagocytes into cells with dendritic morphology and a proinflammatory transcriptional profile but an exhausted allostimulatory activity, Front. Immunol. 10 (2019) 378.

[61] G.D. Brown, J.A. Willment, L. Whitehead, C-type lectins in immunity and homeostasis, Nat. Rev. Immunol. 18 (6) (2018) 374.

[62] A.J. Foster, J.H. Bird, M.S. Timmer, B.L. Stocker, The ligands of C-type lectins, in: S. Yamasaki (Ed.), C-type Lectin Receptors in Immunity, Springer, Tokyo, 2016, pp. 191–215.

[63] J. Ni, Y. Peng, F.-L. Yang, X. Xi, X.-W. Huang, C. He, Overexpression of *clec3a* promotes tumor progression and poor prognosis in breast invasive ductal cancer, OncoTargets Ther. 11 (2018) 3303.

[64] R. Yabe, S. Saijo, Dectin-2 in antimicrobial immunity and homeostasis, in: S. Yamasaki (Ed.), C-type Lectin Receptors in Immunity, Springer, Tokyo, 2016, pp. 3–13.

[65] D. Hartl, P. Latzin, P. Hordijk, V. Marcos, C. Rudolph, M. Woischnik, S. Krauss-Etschmann, B. Koller, D. Reinhardt, A.A. Roscher, Cleavage of CXCR1 on

neutrophils disables bacterial killing in cystic fibrosis lung disease, Nat. Med. 13 (12) (2007) 1423.
[66] N.R. Saha, J.-X. Bei, H. Suetake, K. Araki, W. Kai, K. Kikuchi, H.-R. Lin, Y. Suzuki, Description of a fugu CXC chemokine and two CXC receptor genes, and characterization of the effects of different stimulators on their expression, Fish Shellfish Immunol. 23 (6) (2007) 1324–1332.
[67] M. Cossio-Ayala, M. Domínguez-López, E. Mendez-Enriquez, M. del Carmen Portillo-Téllez, E. García-Hernández, *In vitro* and *in vivo* antimicrobial activity of a synthetic peptide derived from the C-terminal region of human chemokine CCL13 against *Pseudomonas aeruginosa*, Peptides 94 (2017) 49–55.
[68] K. Eslamloo, X. Xue, M. Booman, N.C. Smith, M.L. Rise, Transcriptome profiling of the antiviral immune response in Atlantic cod macrophages, Dev. Comp. Immunol. 63 (2016) 187–205.
[69] C. AndrÚs, F. Acosta, D. Montero, F. Guzmßn, E. Torres, B. Vega, L. Mercado, Synthetic hepcidin from fish: uptake and protection against *Vibrio anguillarum* in sea bass (*Dicentrarchus labrax*), Fish Shellfish Immunol. 55 (2016) 662–670.
[70] L. Cai, J.-J. Cai, H.-P. Liu, D.-Q. Fan, H. Peng, K.-J. Wang, Recombinant medaka (*Oryzias melastigmus*) pro-hepcidin: multifunctional characterization, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 161 (2) (2012) 140–147.
[71] C.A. Álvarez, F. Guzmán, C. Cárdenas, S.H. Marshall, L. Mercado, Antimicrobial activity of trout hepcidin, Fish Shellfish Immunol. 41 (1) (2014) 93–101.
[72] X.-f. Jiang, Z.-f. Liu, A.-f. Lin, L.-x. Xiang, J.-z. Shao, Coordination of bactericidal and iron regulatory functions of hepcidin in innate antimicrobial immunity in a zebrafish model, Sci. Rep. 7 (1) (2017) 4265.
[73] X.-J. Zhang, X.-Y. Zhang, N. Zhang, X. Guo, K.-S. Peng, H. Wu, L.-F. Lu, N. Wu, D.-D. Chen, S. Li, Distinctive structural hallmarks and biological activities of the multiple cathelicidin antimicrobial peptides in a primitive teleost fish, J. Immunol. 194 (10) (2015) 4974–4987.
[74] M. Harboe, G. Ulvund, L. Vien, M. Fung, T. Mollnes, The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation, Clin. Exp. Immunol. 138 (3) (2004) 439–446.
[75] M. Schweiger, R. Schreiber, G. Haemmerle, A. Lass, C. Fledelius, P. Jacobsen, H. Tornqvist, R. Zechner, R. Zimmermann, Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism, J. Biol. Chem. 281 (52) (2006) 40236–40241.
[76] M. Belardinelli, A. Fausto, L. Guerra, F. Buonocore, G. Bongiorno, M. Maroli, M. Mazzini, Lipase and antibacterial activities of a recombinant protein from the accessory glands of female *Phlebotomus papatasi* (Diptera: psychodidae), Ann. Trop. Med. Parasitol. 99 (7) (2005) 673–682.
[77] J.G. Cyster, E.V. Dang, A. Reboldi, T. Yi, 25-Hydroxycholesterols in innate and adaptive immunity, Nat. Rev. Immunol. 14 (11) (2014) 731.
[78] Y. Zhang, L. Wang, X. Huang, S. Wang, Y. Huang, Q. Qin, Fish Cholesterol 25-hydroxylase inhibits virus replication via regulating interferon immune response or affecting virus entry, Front. Immunol. 10 (2019) 322.
[79] A. Yoshimura, M. Ito, S. Chikuma, T. Akanuma, H. Nakatsukasa, Negative regulation of cytokine signaling in immunity, CSH Perspect. Biol. 10 (7) (2018) a028571.
[80] T. Maehr, J.L.G. Vecino, S. Wadsworth, T. Wang, C.J. Secombes, Four CISH paralogues are present in rainbow trout *Oncorhynchus mykiss*: differential expression and modulation during immune responses and development, Mol. Immunol. 62 (1) (2014) 186–198.
[81] C.-Y. Lee, T.-Y. Lai, M.-K. Tsai, Y.-C. Chang, Y.-H. Ho, I.-S. Yu, T.-W. Yeh, C.-C. Chou, Y.-S. Lin, T. Lawrence, The ubiquitin ligase ZNRF1 promotes caveolin-1 ubiquitination and degradation to modulate inflammation, Nat. Commun. 8 (2017) 15502.
[82] P.-H. Lee, B.-G. Kim, S.-H. Lee, J.-H. Lee, S.-W. Park, D.-J. Kim, C.-S. Park, G.D. Leikauf, A.-S. Jang, Alteration in claudin-4 contributes to airway inflammation and responsiveness in asthma, Allergy Asthma Immunol. 10 (1) (2018) 25–33.
[83] U. Repnik, V. Stoka, V. Turk, B. Turk, Lysosomes and lysosomal cathepsins in cell death, BBA-Proteins Proteomics 1824 (1) (2012) 22–33.
[84] M. Peyressatre, C. Prével, M. Pellerano, M. Morris, Targeting cyclin-dependent kinases in human cancers: from small molecules to peptide inhibitors, Cancers 7 (1) (2015) 179–237.
[85] M. Kubo, T. Hanada, A. Yoshimura, Suppressors of cytokine signaling and immunity, Nat. Immunol. 4 (12) (2003) 1169.
[86] E. Mendez-Enriquez, E. Garcia-Zepeda, The multiple faces of CCL13 in immunity and inflammation, Inflammopharmacology 21 (6) (2013) 397–406.
[87] J.M. Kahlenberg, M.J. Kaplan, Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease, J. Immunol. 191 (10) (2013) 4895–4901.