Fish & Shellfish Immunology 47 (2015) 450–460

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Transcriptome immunomodulation of in-feed additives in Atlantic salmon *Salmo salar* infested with sea lice *Caligus rogercresseyi*

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ARTICLE INFO

Article history:
Received 20 May 2015
Received in revised form
31 August 2015
Accepted 2 September 2015
Available online 10 September 2015

Keywords:
RNA-seq
In-feed additives
Head kidney
Skin
Caligus rogercresseyi
Salmo salar

ABSTRACT

One of the most significant threats to the Chilean salmon aquaculture industry is the ectoparasitic sea louse *Caligus rogercresseyi*. To cope with sea lice infestations, functional diets have become an important component in strengthening the host immune response. The aim of this study was to evaluate molecular mechanisms activated through immunostimulation by in-feed plant-derived additives in Atlantic salmon infected with sea lice. Herein, a transcriptome-wide sequencing analysis was performed from skin and head kidney tissues, evidencing that the immune response genes were the most variable after the challenge, especially in the head kidney, while other genes involved in metabolism were highly expressed individuals fed with the immunostimulants. Interestingly, defensive enzymes such as Cytochrome p450 and serpins were down-regulated in infested individuals, especially in skin tissue. Additionally, MHC-I and MHC-II genes were differentially expressed after the incorporation of the in-feed additives, giving some cues about the protection mechanisms of plant-derived compound as immunostimulants for infested salmons. This is the first published study that evaluates the transcriptomic response of sea lice-infested Atlantic salmon fed with in-feed additives.

1. Introduction

For the last decade, Chile has been a leading worldwide aquaculture producer, second only to Norway. However, this industry depends in large part on Atlantic salmon (*Salmo salar*) farming, which reached its peak in 2008 [1]. Despite considerable production efforts, this industry has been significantly impacted by sanitary issues, resulting in a multidimensional crisis [2]. Currently there is serious concern about the presence of parasites, particularly sea lice, and the sanitary and economic consequences of these in salmon farming [3].

Caligidosis is the sea lice parasitosis affecting the Chilean industry. Caused by *Caligus rogercresseyi*, this ectoparasite has caused high economic losses in recent years [4]. Most of these costs are due to the treatments used to control this disease, which generally consist in chemical compounds that attack the parasite's biological processes, such as molting and nervous system functions [5]. Additionally, these antiparasitic drugs have diverse environmental impacts on non-target species and marine habitats [6–10], and some are highly persistent in the marine environment [11]. Considering the above, there is an urgent need to develop novel control strategies for sea lice infestations in salmon farms.

A diversity of defense responses to marine ectoparasitic copepods have been studied over the last decades in various fish species, including physiological, cellular, and molecular responses [12]. The Atlantic salmon has evidenced varied physiological responses to Northern hemisphere sea lice (*Lepeophtheirus salmonis*) infestations, such as increases in mucus production, cell mucus proliferation, tissue erosion, and inflammation of the affected tissues [13–16]. These responses are modulated depending on fish welfare and the lifecycle stage of the parasite. Short-term sea lice exposure assays produced stress responses in *S. salar*, increasing cortisol levels and the transcription of genes involved in coping

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http://dx.doi.org/10.1016/j.fsi.2015.09.009
1050-4648/

with acute stress [17,18], and when the infestation period was extended, secondary responses were triggered involving energy metabolism and hydromineral balance [19] or the impairment of hormonal, electrolyte, and hematocrit levels [20]. Regarding *C. rogercresseyi*, a recent study cited some physiological responses of *S. salar* after infestation with different parasitic loads, finding that as few as six parasites could trigger detrimental effects to the welfare of farmed fish, which is lower than the threshold load determined by Chilean regulatory authorities for salmon farms [21].

To understand the modulation of host-parasite responses, researchers have focused on the regulation of defense mechanisms in fish after sea lice infestation at a molecular level. A microarray study in specific tissues (i.e. intact skin, damaged skin, spleen, head kidney, and liver) evaluated the gene expression levels of Atlantic salmon after 22 and 33 days of *L. salmonis* infestation, finding that various stress response genes are up-regulated at the initial stages while immune related genes are regulated during subsequent stages [22]. Another transcriptomic assay, also with microarray analysis, suggested that the innate and adaptive immune systems of Atlantic salmon are modulated specifically by the transitional phase of parasite development, from the copepodid (infective stage) to chalimus (juvenile stage) [23]. Moreover, a recent study showed that the defense response of Atlantic salmon to *L. salmonis* also depends on the genetic line of hosts, where families present different modulations of immune related and detoxification genes depending on the susceptibility/resistance of certain genotypes [24].

Diverse biotechnological products have been applied as part of disease prevention and control strategies in response to the sanitary issues of the aquaculture industry in recent years, including vaccines, antibiotics, and dietary supplements, among others [25]. In this context, diets supplemented with additives of different natures is a high-potential solution owing to its effectiveness in more safely controlling diseases than antibiotics or chemotherapeutics [26]. In salmonids, information regarding the effectiveness of dietary solutions to reduce sea lice infestation levels is still limited. At present, functional diets including immunostimulants or essential oils derived from natural extracts have been tested in Atlantic salmon, with results evidencing up to a 20% decrease in *L. salmonis* infestation [27]. Nonetheless, the regulatory mechanisms of this stimulation at a molecular level remain unknown in Atlantic salmon infested with *C. rogercresseyi*.

Given the importance of understanding the mechanisms behind immunomodulation during sea lice infestation and immunostimulant treatment in salmonids, the aim of this study was to evaluate the transcriptome modulation of in-feed additives (immunostimulant + anti-attachment compounds) for sea lice-infested *S. salar*. In specific, both immunostimulant and anti-attachment compound corresponded to natural extracts from plants. The immunostimulation of this plant extract cause cellular responses found in general plant immunostimulants, such as leukocyte migration, production of innate immune response proteins (interleukins, chemokines, lysozymes) and increase in phagocytosis activity [28,29]. The selected anti-attachment compound was a natural plant extract suggested to have repellent effect in sea lice (unpublished data).

2. Materials and methods

2.1. Experimental groups and sampling

One hundred and eighty Atlantic salmon (*S. salar* ~300 g each) were acclimated for 14 days in separate tanks (n = 30 fish per tank) with identical conditions of optimal salinity, dissolved oxygen, and temperature. One treatment (3 replicates) was fed with a commercial basal diet (EWOS, Chile) without additives and a second treatment (3 replicates) was fed the same diet supplemented with 1% immunostimulant and 3% of anti-attachment compound (EWOS Innovation, Chile). Both immunostimulant and anti-attachment were obtained from extraction of plant-derived natural compounds. After 21 days these two groups (control diet and additives-supplemented diet) were infested with *C. rogercresseyi* (35 copepodids/fish). The remaining group was left uninfested as a control condition. All groups were sampled 15 days after sea lice infestation (Fig. 1A). For sampling, fish were killed with an overdose of benzocaine and examined for sea lice. Skin and head kidney tissue were dissected from all specimens, fixed in RNA Later® solution (Ambion®, Life Technologies™, USA), and stored at −80 °C.

2.2. RNA isolation, sequencing, and bioinformatics processing

Samples of total RNA were extracted using the RiboPure™ Kit (Ambion, Life Technologies™, USA) according to the manufacturer's instructions for skin and head kidney tissues. RNA concentration and purity were calculated using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies™, USA), and quality of isolated RNA was measured in a TapeStation 2200 using the R6K Reagent Kit according to the manufacturer's instructions (Agilent Technologies Inc., Santa Clara, CA, USA), and samples with a RIN over 8.0 were selected. Subsequently, cDNA libraries were constructed for each experimental group using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA). Tissues were processed separately, resulting in nine different libraries constructed from pools of samples (one for each tissue for each group). Three biological replicates for each library were sequenced by the MiSeq (Illumina®, USA) platform using sequenced runs of 250 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

The data generated through RNA sequencing were processed using the CLC Genomic Workbench software (version 8.0, CLC Bio, Aarhus, Denmark). Briefly, raw data from each library were trimmed separately and read sequences were then *de novo* assembled into a single file encompassing the three experimental groups for each tissue evaluated using the following settings: mismatch cost = 2, insert cost = 3, minimum contig length = 400, similarity = 0.8, and trimming quality score = 0.05.

2.3. Transcriptome expression analyses

First, a global transcriptome expression evaluation was performed for all expressed data in each tissue and group. To conduct these analyses, RNA-seq tests were performed for the raw trimmed data of each dataset using *de novo* assembled contigs as references from skin and head kidney tissue, respectively. RNA-seq settings were a minimum length fraction of 0.6 and a minimum similarity fraction (long reads) of 0.5. Gene expression was measured and normalized by RPKM (reads per kilobase per million of mapped reads) calculations in each dataset. Then, specific RNA-seq experiments were set up according to different gene expression comparisons, either by tissues or groups. Comparisons of expression data for each tissue consisted in a “Feed control,” a comparison between uninfested and infested individuals fed with the control diet; and an “In-feed additives,” a comparison between the infested groups, one of which was fed with in-feed additives. Specific experiments were normalized by quantiles to calculate fold-change values, which, as multiple pair comparisons, were visualized by constructing volcano plots. One volcano plot was constructed based on Baggerley's test of proportion data for both the “Infestation

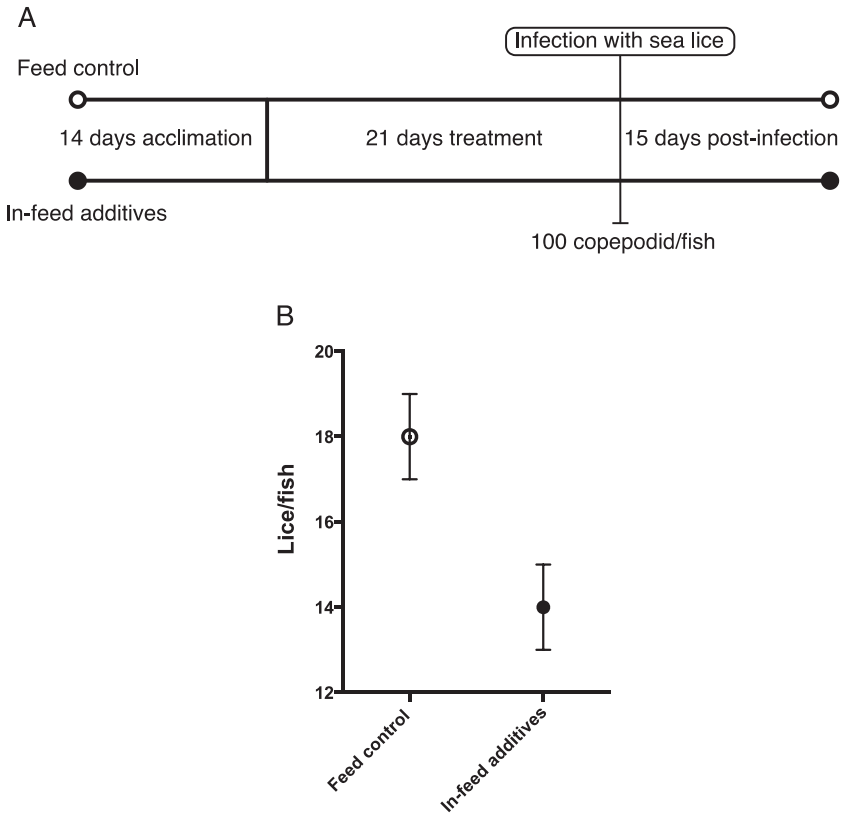

Fig. 1. Timeline of overall study and sea lice infestation densities. (A) Infested-Atlantic salmon fed with control feed and in-feed additives. Lines represent the acclimation, treatment and post-infestation periods. The infestation point was 35 days after started the trials and the sample point was 15 days post-infection. (B) Sea lice infestation density in Atlantic salmon fed with control diet and supplemented with additives.

effect" and "Immunostimulant effect" in both tissues. These analyses were FDR-corrected, and the significant threshold for transcriptomic expression data was set at $>\log_2(|2|)$ for fold-change values and $\log_{10}(2)$ for p-values.

Additionally and to obtain higher functionality evaluation transcriptomic data were annotated in the KEGG database. The KAAS annotation web-based tool was used to compare the *de novo* assembly from *S. salar* with the KEGG public database. From all the enriched pathways gene expression analysis was carried out for some pathways considered to be of high biological relevance due to the experiment nature. Since this work evaluates the effect of the infestation by the copepod and the effect of an immunostimulant diet in two immune related organs we considered important to focus on pathways related to immune response signaling and selected cellular enzymes. Furthermore, since it is known the deleterious effect of lice infestations on overall health and energy balance of infected fish [30], we considered important to evaluate the major energy related metabolic pathways, as well as the wound healing capacity since it would be a proxy to the host's capacity to recovery from the typical clinical damages imposed by lice infestation. All the contigs from immune response signaling (Toll-like receptor signaling pathway, NOD-like receptor pathway, Chemokine signaling pathway, Antigen processing and presentation, Drug metabolism, Cell adhesion molecules, among others) and metabolic processes (e.g. Glycolysis, gluconeogenesis, TCA cycle, pentose phosphate pathway, insulin signaling pathway, fatty acid degradation and biosynthesis, among others) were extracted and mapped to datasets for each group. Finally, RNA-seq analyses were performed from these selected contigs with the same aforementioned settings. Furthermore, visualization of gene expression data for each tissue and experimental group was performed by constructing heat maps based on hierarchical clustering of expressed data in the CLC software. Then, fold-change values were calculated and extracted to perform statistical analyses, with the aim of identifying the most significantly expressed genes.

2.4. Statistical analysis

Fold-change differences for contigs active in the "Feed control" and "In-feed additives" groups were evaluated for each tissue separately with the Baggerley's test and accepted when $p < 0.01$ [31]. Regarding the expression analysis of selected pathways after RNA-Seq analysis a filtering workflow was applied to identify the most relevant genes. The contigs expressing more than 1 fold change in each group were retained. To further select the differentially expressed contigs and retain those most relevant in the two effects studied (control and additives), a Principal Component Analysis (PCA) was performed on the expression levels (in RPKM) of each contig. This was executed as an ordination technique for variable (contigs) reductions to reveal the underlying structure of the dataset and reveal the most significant contigs while accounting for the studied effects. The PCA was performed by tissue and by effect. Principal components were accepted when their eigenvalues were >1 following the Kaiser criterion, and the contigs were considered relevant when their load in the PCA was >0.8 as their contribution for observed sub-experiment data set structure was considered highly significant (maximum is 1). Based on this criteria the 10 more significant contigs that loaded the differences between the groups in study (feed control and in-feed additives) were finally retained to be interpreted as the most important variables representing the effect of the groups on the chosen pathways.

Table 1
Summary of *Salmo salar* RNA-sequencing throughput.

	Count	Average length (bp)	Total bases
Skin			
Reads	36,168,876	186.24	6,735,948,008
Matched reads	24,565,662	184.62	4,535,293,834
Non-matched reads	11,603,214	189.66	2,200,654,174
Contigs	161,651	572.45	92,592,901
Head kidney			
Reads	34,381,498	195.25	6,777,206,916
Matched reads	22,208,380	190.48	4,336,148,946
Non-matched reads	12,173,118	200.53	2,441,057,970
Contigs	130,931	595.97	77,925,791
Skin + head kidney			
Reads	70,550,374	191.12	13,513,154,924
Matched reads	46,774,042	188.21	8,871,442,780
Non-matched reads	23,776,332	195.53	4,641,712,144
Contigs	292,432	584.45	170,518,692

3. Results

3.1. Infestation densities and global transcriptome response

Infestation density at 15 days post-infestation was the highest in Atlantic salmons fed with the control diet (18 ± 1.2 lice/fish); whereas, infestation density was significantly reduced ($p < 0.01$) in fish fed with an additive-supplemented diet (Fig. 1B).

Transcriptomic data for each group were obtained from MiSeq runs, yielding almost 70.5 million 191 base pair reads when considering the combined data from all sequencing runs. Throughput sequencing of *de novo* assembled reads resulted in 161,651 and 130,931 contigs, with an average length of 573 and 595 base pairs for skin and head kidney transcriptomes, respectively (Table 1). The reads of each experimental group were successfully mapped into the *de novo* assembly used as reference for RNA-seq analyses.

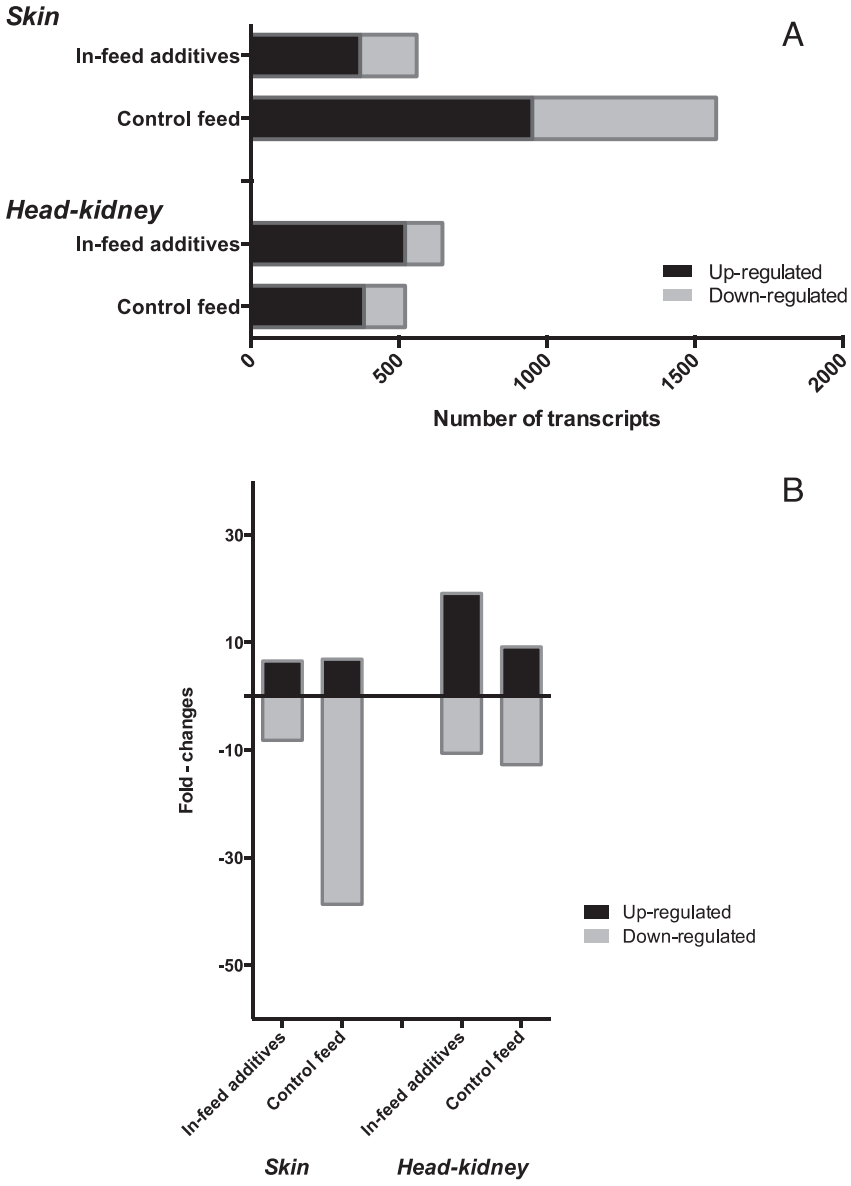

Fig. 2. Skin and head kidney transcriptome response. (A) Number of transcripts up or down-regulated according Baggerley's test (p-values > 0.01 (y-axis) and $>\log_2|2|$) from Atlantic salmon fed with control feed and in-feed additives. (B) Fold-changes values from up or down-regulates transcripts identified from skin and head kidney transcriptome.

Skin transcriptome expression displayed a wide variety of genes with diverse biological functions. In the case of the "Feed control," 950 different contigs were significantly up-regulated after sea lice infestation, while 621 contigs were down-regulated. Regarding "In-feed additives" in skin expression, 368 contigs were up-regulated and 191 contigs were down-regulated. In the Atlantic salmon head kidney, sea lice exposure in the "Feed control" up-regulated 381 contigs and down-regulated 139. For head kidney analysis considering "In-feed additives," 320 contigs were significantly up-regulated (Fig. 2A). Some significantly up and down-regulated genes by each tissue are shown in supplementary figures S1–S4.

The assessment of fold-change values between tissues and groups evidenced that all down-regulated transcripts were roughly 10-fold changes, except for in the skin of Atlantic salmon fed with the control diet, in which values evidenced up to 38-fold changes. Interestingly, up-regulated transcripts in the head kidney from individuals fed with diets supplemented with additives displayed nearly 20-fold changes, whereas "Feed control" fish evidenced roughly 9-fold changes (Fig. 2B).

3.2. Cluster analysis of skin and head kidney transcriptomes

Global gene expression profiles represent a useful tool to understand the effects of functional diets on Atlantic salmon transcriptomes. Considering this, differential gene expressions between the "Feed control" and "In-feed additives" groups were analyzed using RNA-seq analysis (Fig. 3). The RPKM values were visualized with a heatmap based on a hierarchical clustering of features (CLC Genomic Workbench nomenclature), while distance was estimated by the Manhattan method. Statistical analysis confirmed that the infested Atlantic salmon fed with in-feed additives modified their transcriptomes, evidencing the formation of six and four clusters of transcripts with different patterns of expression for the skin and head kidney, respectively (Fig. 2). With regards to skin tissue,

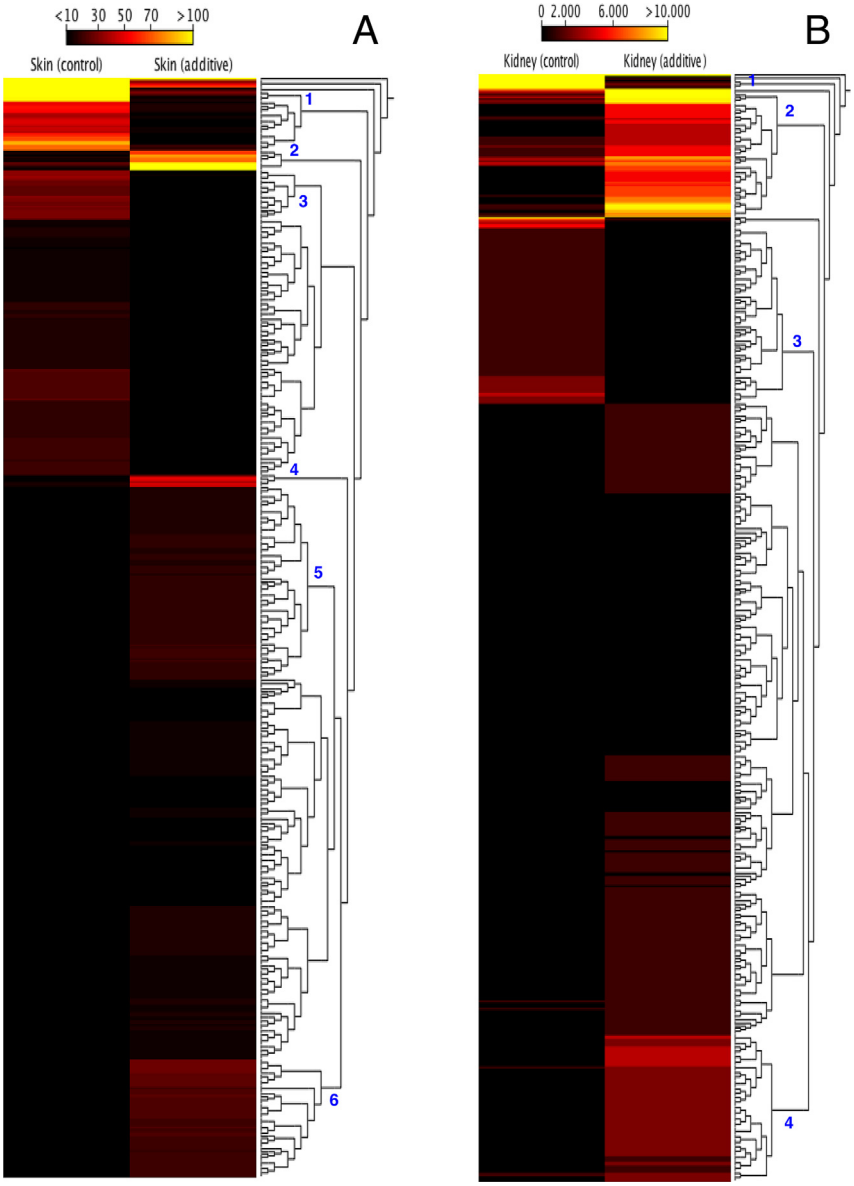

Fig. 3. Heat maps of highly-regulated transcripts from infested-Atlantic salmon feed with control diet and in-feed additives. (A) Skin transcriptomes. (B) Head kidney transcriptomes. Clusters are indicated with numbers and transcript abundance is represented as RPKM values and color scales show relative transcript expression.

clusters 2, 4, 5, and 6 were composed of up-regulated transcripts in the group of fish fed with diets supplemented with immunostimulants and anti-attachment compounds (Table 2). Interestingly, cluster 1 was highly down-regulated, annotating for genes related to skin tissue, such as *Epidermis-type lipoxigenase*, *Keratin-type I*, and *Mucin-5B* (Fig. 3A). Moreover, head kidney transcriptomes comprised two up-regulated clusters, which were mainly annotated for immune-related genes, such as *MHC-Class I* in cluster 2 (Fig. 3B). It is important to note that some genes related to blood iron regulation, such as *Haptoglobin* and *Hemoglobin embryonic subunit alpha*, were down-regulated in infested Atlantic salmon fed with in-feed additives (Table 3).

3.3. Signaling pathways of relevant genes

From the sub-data set extracted from the selected pathways of interest the contigs with more than 1 fold expression for the effects tested were extracted. This resulted in 439 contigs in skin and 427 contigs in kidney. The principal component analysis based on the expression of those contigs for each observation revealed the differences promoted by the effects studied (Fig. S5), identifying data structure with no overlapping between observations principal scores. The contigs (or variables) significantly loading the separations between the observations before and after infestation (infestation effect) and with and without immunostimulants (In-feed additives effect) were selected as described above and the fold change of the 10 most significant for each effect in each tissue is presented in Table 4. Immune-related transcripts exhibited changes in expression levels after exposure to *C. rogercresseyi* in the head kidney and skin of infested fish. Most of the differentially expressed genes were involved in the inflammatory response, wound healing processes, and cell proliferation. Among these genes were *chemokines*, *c-Fos*, *mitogen-activated protein kinase*, and *nuclear transcription factor*, which were up-regulated in the skin of the control-feed infested group. For the immunostimulated group, these genes were activated, with the exception of *CD4* gene, which was down-regulated. In general, the selected immune related genes were down-regulated in the head kidney after sea lice exposure, with the most significant changes in *nuclear transcription factor*, *CD4*, and *c-Fos*. However, no changes were observed in these transcripts as

Table 2
Cluster expression analysis from skin in infested Atlantic salmon fed with in-feed additives.

Feature ID	Fold change	P-value	Annotation	Function (GO)
Cluster 1				
Contig_0014589	-4.30	1.79E-14	MHC class I antigen [Salmo salar]	immune response
Contig_0013573	-4.60	1.60E-06	proline dehydrogenase 1, partial [Salmo salar]	proline dehydrogenase activity
Contig_0013318	-4.66	7.90E-10	Mucin-5B [Gallus gallus]	structural
Contig_0011177	-4.74	1.76E-06	Dual specificity protein phosphatase 5 [Salmo salar]	protein binding
Contig_0018884	-4.91	3.89E-15	Pyruvate dehydorgenase kinase isozyme 2, mitochondrial precursor [Salmo salar]	kinase activity
Contig_0017731	-5.01	2.59E-10	c-Fos protein [Oncorhynchus masou]	chromatin binding
Contig_0016596	-7.62	1.79E-08	Epidermis-type lipoxygenase 3 [Salmo salar]	iron ion binding
Contig_0006207	-9.20	3.58E-12	Keratin, type I cytoskeletal 20 [Salmo salar]	structural
Contig_0014546	-12.88	0	trout C-polysaccharide binding protein 1, isoform 1 [Oncorhynchus mykiss]	immune response
Cluster 2				
Contig_0012862	40.71	0	myosin heavy chain, partial [Oncorhynchus keta]	ATP binding
Contig_0010357	11.86	0	myosin heavy chain, partial [Oncorhynchus mykiss]	ATP binding
Contig_0013736	7.95	3.22E-12	MHC class I antigen, partial [Salmo salar]	antigen binding
Contig_0014119	7.62	6.92E-13	coiled-oil transcriptional coactivator b [Salmo salar]	transcription
Contig_0018835	6.52	8.88E-16	heat shock protein hsp90 [Oncorhynchus tshawytscha]	ATP binding
Contig_0017763	5.84	2.68E-09	coiled-oil transcripctinaol coactivator b [Salmo salar]	transcription
Contig_0013595	4.11	2.47E-12	MHC class I antigen, partial [Salmo salar]	immune response
Cluster 3				
Contig_0013838	-4.12	1.59E-04	Sperm acrosome membrane-associated protein 4 precursor [Salmo salar]	cell adhesion
Contig_0003744	-4.14	8.45E-04	Sodium-couple neutral amino acid transporter 2 [Salmo salar]	structural
Contig_0005631	-4.14	2.34E-03	Heterogeneous nuclear ribonucleoprotein A0 [Salmo salar]	nucleotide binding
Contig_0005985	-4.14	2.73E-03	Nattectin precursor [Salmo salar]	carbohydrate-binding activity
Contig_0007721	-4.14	5.46E-03	NADP-dependent malic enzyme, mitochondrial precursor [Salmo salar]	malate dehydrogenase activity
Contig_0007264	-4.14	6.09E-05	Trafficking kinesin-binding protein 1 [Homo sapiens]	transport
Contig_0005171	-4.60	6.33E-04	Apoptotic chromatin condensation inducer in the nucleus [Mus musculus]	nucleotide binding
Contig_0000862	-4.60	6.32E-03	DNA-damage-inducible transcript 4-like [Salmo salar]	transduction
Contig_0006388	-4.60	3.89E-03	Protein phosphatase PTC7 homolog [Homo sapiens]	metal ion binding
Contig_0006772	-4.93	6.80E-05	succinate dehydrogenase complex subunit A flavoprotein [Salmo salar]	heme binding
Contig_0005305	-5.18	7.85E-04	Keratin, type I cytoskeletal 20 [Salmo salar]	structural
Contig_0003037	-5.37	8.03E-04	Solute carrier family 25 member 33 [Salmo salar]	transmembrane transport
Contig_0000783	-5.75	9.24E-04	Twist-related protein 2 [Salmo salar]	transcription
Contig_0003315	-5.75	4.33E-03	Cathepsin H precursor [Salmo salar]	peptidase activity
Contig_0001449	-6.90	9.93E-03	Myocyte-specific enhancer factor 2C [Bos taurus]	DNA binding
Contig_0005227	-6.90	2.91E-03	Transforming acidic coiled-coil-containinig protein 1 [Homo sapiens]	cell proliferation
Contig_0004567	-6.90	1.33E-05	Glucosidase 2 subunit beta [Homo sapiens]	calcium ion binding
Contig_0002977	-6.90	8.59E-04	Nucleoside diphosphate-linked moiety X motif 17 [Danio rerio]	hydrolase activity
Contig_0006127	-8.05	1.43E-03	haptoglobin fragment 2, partial [Oncorhynchus mykiss]	endopeptidase activity
Contig_0001089	-9.20	1.27E-03	Titin [Homo sapiens]	ATP binding
Contig_0003364	-9.20	4.70E-03	SUMO-activating enzyme subunit 2 [Salmo salar]	ATP binding
Contig_0002691	-11.5	7.14E-04	reverse transcriptase-like protein [Salmo salar]	reverse transcription
Contig_0001734	-13.8	6.07E-05	follistatin b1 [Oncorhynchus mykiss]	protein binding
Contig_0002695	-13.8	1.49E-04	Transketolase [Salmo salar]	metal ion binding
Contig_0002270	-16.1	2.10E-05	N-lysine methyltransferase SETD8-A [Danio rerio]	N-methyltransferase activity

Feature ID	Fold change	P-value	Annotation	Function (GO)
Cluster 4				
Contig_0009117	14.78	4.80E-10	Parvalbumin alpha [Salmo salar]	calcium ion binding
Contig_0015893	9.89	8.50E-09	Myosin-binding protein C, fast-type [Homo sapiens]	structural
Contig_0015477	4.43	6.19E-06	Myosin-binding protein C, fast-type [Homo sapiens]	structural
Cluster 5				
Contig_0006212	8.26	7.00E-04	Ras-related protein R-Ras [Mus musculus]	GTP binding
Contig_0006814	7.39	2.00E-03	Serine/threonine-protein kinase/endoribonuclease ire-1 [Caenorhabditis elegans]	ATP binding
Contig_0006526	6.95	3.00E-03	U3 small nucleolar RNA-associated protein 15 homolog [Salmo salar]	rRNA processing
Contig_0006479	6.52	5.00E-03	RING finger protein 39 [Macaca mulatta]	zinc ion binding
Contig_0006920	6.52	5.00E-03	immunoglobulin light chain, partial [Salmo salar]	immune response
Contig_0005139	6.09	4.00E-03	complement factor H precursor [Oncorhynchus mykiss]	protein binding
Contig_0005070	5.65	6.00E-03	insuline-like growth factor binding protein 3 paralog A1 [Salmo salar]	protein binding
Contig_0005627	5.65	8.00E-03	Signalosome complex subunit [Danio rerio]	metal ion binding
Contig_0004506	5.22	9.00E-03	Transmembrane and ubiquitin-like domain-containing protein 2 [Salmo salar]	structural
Contig_0004783	5.22	1.00E-02	Amidophosphoribosyltransferase precursor [Salmo salar]	metal ion binding
Contig_0003223	4.78	9.00E-03	Alkaline phosphatase [Salmo salar]	alkaline phosphatase activity
Contig_0008528	4.56	5.00E-03	transmembrane protein 111 [Oncorhynchus mykiss]	structural
Contig_0007253	4.13	7.00E-03	Plectin [Cricetulus griseus]	structural
Cluster 6				
Contig_0006769	13.91	3.32E-06	Glutamate decarboxylase-like protein 1 [Salmo salar]	carboxy-lyase activity
Contig_0007152	10	1.94E-04	Myozenin-1 [Salmo salar]	protein binding
Contig_0007113	9.56	2.95E-04	MHC class I antigen [Salmo salar]	antigen binding
Contig_0003750	7.39	3.80E-04	S-adenosylmethionine synthetase isoform type-1 [Salmo salar]	ATP binding
Contig_0009951	6.74	3.50E-04	Phosphotriesterase-related protein [Salmo salar]	hydrolase activity
Contig_0009925	6.52	4.48E-04	hypotetical protein [Oncorhynchus mykiss]	
Contig_0008708	6.3	3.48E-04	Myoferlin [Mus musculus]	phospholipid binding
Contig_0008574	6.09	4.58E-04	homeobox protein HoxD12aa [Salmo salar]	sequence-specific DNA binding
Contig_0006042	5.87	1.55E-04	mitochrondrial-processing peptidase subunit alpha [Bos taurus]	proteolisis
Contig_0005468	5.22	3.94E-04	DNA polymerase beta [Danio rerio]	DNA binding
Contig_0008720	5	2.90E-03	WD repeat-containing protein 82 [Salmo salar]	protein binding
Contig_0007476	4.2	9.60E-04	Growth-regulated alpha protein phosphatase PP1-gamma catalytic subunit [Salmo salar]	protein binding
Contig_0009073	4.2	1.93E-03	serine/threonine-protein phosphatase PP1-gamma catalytic subunit [Salmo salar]	phosphoprotein phosphatase activity
Contig_0005321	4.13	3.57E-03	anaphase-promoting complex subunit 7 [Salmo salar]	protein binding

compared to the immunostimulated group. On the other hand, while the MHC-II complex transcript did not show significant differences in infestation-only comparisons, this was down-regulated as compared to the immunostimulated group. Regarding wound healing processes, an up-regulation of transcriptomic levels in the skin and kidney of infested-only fish as compared to control fish was observed, but two of these genes were down-regulated in both tissues by the inclusion of immunostimulants in the salmon diet (*growth factor receptor-bound protein-2* and *plasminogen activator inhibitor 1 precursor*). *C. rogercresseyi* infestation itself had antagonistic effects on two enzymes participating in arachidonic acid metabolism, since it up-regulating *epidermis-type lipoxygenase 3* but down-regulating *prostaglandin E synthase 3* in both tissues, but immunostimulation counteracted these effects (only in skin for *epidermis-type lipoxygenase 3*). Furthermore, other enzymes involved in defense response were studied, including detoxification enzymes, proteins involved metabolism of xenobiotics, and other kinases and proteases that are components of relevant biological pathways. Overall, the most significant enzymes among these groups exhibited decreased expression levels after exposure to *C. rogercresseyi*, especially in skin tissue (Table 4). The most down-regulated genes after parasite exposure alone were *cytochrome p450* and some serpins such as *alpha-1 antiproteinase-like* and *antithrombin* proteins. In contrast, the gene transcription patterns of these changed when comparing against the in-feed additive group, showing up-regulation in some cases. In skin tissue, among the contigs that changed expression patterns were *UGT glucuronosyltransferase*, involved in the metabolism of xenobiotics, *proline serine-threonine phosphatase-interacting protein 2*, *heat shock protein 47*, which is a serine proteases inhibitor, and *tyrosine-protein kinase BTK putative*.

Most of the genes involved in the energy-producing metabolic pathways (Glycolysis and TCA cycle) were affected by lice infestation alone, except for *alcohol dehydrogenase 1-like* and *malate dehydrogenase 1*, which were up-regulated in the head kidney and skin, respectively. The immunostimulant lessened these effects to some extent by increasing the expression of the selected genes in the head kidney. In both tissues, the insulin signaling route participant suppressor of the *cytokine signaling 3* gene was up-regulated by parasite infestation, whereas immunostimulation down-regulated this gene.

4. Discussion

The obtained results evidenced a noticeable change in expression levels of host *S. salar* after infestation with *C. rogercresseyi*, with a wider transcriptomic response in skin tissue. This is consistent with the infestation mechanism used by this parasite, which directly affects the first barrier of defense in fish, the mucus and the defense proteins in the skin. Similar results were obtained in the

Table 3
Cluster expression analysis from the head kidney in infested Atlantic salmon fed with in-feed additives.

Feature ID	Fold change	P-value	Annotation	Function (GO)
Cluster1				
Contig_0028370	4.92	1.0E-34	MHC class I antigen, partial [Salmo salar].	immune response
Contig_0045951	6.56	3.3E-29	Band 3 anion exchange protein [Oncorhynchus mykiss]	ion transport
Contig_0050259	7.53	6.6E-33	Band 3 anion exchange protein, partial [Salmo salar].	ion transport
Contig_0031471	-1.43	1.7E-92	Putative uncharacterized protein [Saccharomyces cerevisiae]	
Contig_0029743	-9.96	4.4E-172	Protein TAR1 [Kluyveromyces lactis]	mitochondrial stability
Contig_0029501	-91	6.5E-212	60S ribosomal protein L36a [Salmo salar].	structural
Contig_0034685	-8.38	1.2E-174	Protein NLRC3 [Mus musculus]	immune response
Cluster2				
Contig_0041225	40.58	0	Protein TAR1 [Saccharomyces cerevisiae]	cellular respiration
Contig_0048816	37.82	0	MHC class I, partial [Salmo salar].	immune response
Contig_0038163	34.4	0	MHC Class I, partial [Salmo salar].	immune response
Contig_0029126	20.07	0	MHC class II antigen beta chain, partial [Salmo salar].	immune response
Contig_0032192	7.48	0	MHC class I heavy chain precursor, partial [Oncorhynchus mykiss].	immune response
Contig_0013888	7.35	0	MHC class I heavy chain precursor [Oncorhynchus mykiss].	immune response
Contig_0034980	7.29	1.8E-13	myosin regulatory light chain 2 [Salmo salar].	ATP binding
Cluster3				
Contig_0048021	-4.3	7.9E-09	Haptoglobin [Oncorhynchus mykiss].	hemoglobin binding
Contig_0044448	4.75	1.4E-06	Aquaporin FA-CHIP [Salmo salar].	transporter activity
Contig_0005808	5.83	3.0E-07	Ig kappa chain V-IV region B17 precursor [Salmo salar].	immune response
Contig_0022761	7.66	7.6E-09	Hemoglobin embryonic subunit alpha [Salmo salar]	iron ion binding
Contig_0026741	-10.81	1.2E-18	Immunoglobulin heavy chain variable region, partial [Salmo salar].	immune response
Cluster4				
Contig_0019118	54.59	2.8E-10	Protein TAR1 [Kluyveromyces lactis]	mitochondrial stability
Contig_0025537	25.06	7.4E-11	Protein TAR1 [Saccharomyces cerevisiae].	mitochondrial stability
Contig_0038665	12.59	1.3E-15	parvalbumin like 1 [Salmo salar].	calcium ion binding
Contig_0020998	8.5	3.9E-09	Proteasome subunit beta type-9 precursor [Salmo salar].	endopeptidase activity
Contig_0044873	5.65	3.4E-08	Proteasome subunit beta type-8 precursor [Salmo salar].	endopeptidase activity

same host species infested with the Northern hemisphere sea louse *L. salmonis*, where higher amounts of differentially expressed genes were found in skin tissue than in the liver, head kidney, and spleen [22].

In regards to specific pathways, immune-related genes with significant differences after parasite exposure corresponded to MAPK signaling pathway components, especially pro-inflammatory regulators. This response has been related with damage in tissues caused by sea lice in salmonids and other fish species. Moreover, the most resistant individuals have a greater production of pro-inflammatory genes than susceptible fish [22]. In Atlantic salmon infested with *L. salmonis*, decreased expression levels of chemokines have also been described [22,23]. Consistent with this previous research, the current analyses found that the expression levels of chemokine genes decreased after *C. rogercresseyi* infestation in *S. salar* [23]. In turn, the activation of MAPK signaling components in the present study could be indicative of apoptotic or cellular proliferation triggered by the infestation process. This can be compared with a previous study reported by Braden et al. [32], where similar results were observed in *S. salar* infested by *L. salmonis*. Other defense-response enzymes showed a general decrease in expression levels after infestation, mainly in the skin tissue. This is concordant with the infestation mechanism of the salmon louse, which has been suggested to involve an immunosuppression mechanism in host fish and the eventual inactivation of other host-defense responses [33,34]. Serpins were also down-regulated, suggesting that the inactivation of *C. rogercresseyi* proteases was also affected in infested fish. One forthcoming approach aimed at understanding this interaction would be comparing at the transcriptomic level these genes from hosts with proteases in sea lice, or even with serpins in the parasitic species, which were recently characterized [30].

Interestingly, *MHC-II*, which has a role in antigen presentation to trigger the adaptive immune response, showed decreased expression levels in the head kidney of individuals in the present study. Similar results were found after a 3 day *L. salmonis* infestation, with the difference that non-immunostimulated fish in the present experiment did not have significant differences as compared to non-infested control specimens [35]. Inhibition in the head kidney of immunostimulated fish may explain the decreased expression levels of CD4 markers since CD4 lymphocytes interact with MHC II during the antigen presentation process. Nonetheless, after 30 days of exposure to *L. salmonis*, Atlantic salmon have evidenced increases in CD4 marker levels [22], which suggests that the adaptive immune response might occur later and cannot be observed after just 15 days of exposure to the Southern hemisphere sea louse *C. rogercresseyi*. Regarding *MHC I*, up-regulated levels were found after sea lice infestation in the present study, which is opposite to that found in the previously cited research of *L. salmonis*. Further research should compare the molecular signals activated by exposure to both parasites considering that the physiological and epidemiological characteristics of each species could result in different signaling activation in affected salmons, which in turn could mean requiring different treatment methods.

Wound healing and epidermal regeneration are important processes in the pathological response and recovery, but these processes are especially important in parasitic infestations, such as with sea lice. Copepod attachment to the host is with a frontal filament that penetrates the skin, thus producing wounds in addition to the irritating effects of the feeding movements around the attachment zone [29]. These processes have several participating genes, but in this study three were found to have relevant roles in the observed effects. The *growth factor receptor-bound protein 2* (*GRB2*) gene codes for an adapter protein that

Table 4
Gene transcription levels for the most relevant contigs selected for biological processes of interest (continuation).

Annotation	e-value	Pathway	*Feed control*		*In-feed additives*	
			Skin	Head kidney	Skin	Head kidney
Immune/defense response genes						
Growth factor receptor-bound protein 2	4.00E-130	Angiogenesis/Wound healing	4.92	2.2	−1.56	−1.01
Angiopoietin-1	5.00E-139	Angiogenesis/Wound healing	3.55	1	1.13	1
Plasminogen activator inhibitor 1 precursor	0	Angiogenesis/Wound healing	1.77	3.18	−1.95	−2.51
Protein kinase A	1.00E-77	Apoptosis/Other signaling	1	−179.99	0	−1.31
Beta-adrenergic receptor kinase 2	0	Beta-adrenergenic activation	−1.08	−4459.45	1.16	−1.15
Chemokine receptor 7	0	Chemokine signaling	0	−49.2	−2.24	−1.37
Chemokine receptor type 9	0	Chemokine signaling	1.08	1633.68	−4.66	−1.63
Chemokine receptor type 9-like	1.00E-118	Chemokine signaling	4.67	−4305.37	−6.21	1.38
Phosphatidylinositol 4.5-bisphosphate 3-kinase	2.00E-151	MAPK signaling	−1.61	−3047.11	1.01	1.61
Phosphatidylinositol 4.5-bisphosphate 3-kinase	0	MAPK signaling	0	796.67	1.01	−1.31
cAMP-dependent protein kinase catalytic subunit beta	0	MAPK signaling	−1.43	1167.34	2.1	−1.24
Mitogen-activated protein kinase kinase kinase 7-like	0	MAPK signaling	3.27	82.1	−8.7	−2.94
c-Fos protein	2.00E-163	MAPK signaling	1.43	−534.69	−4.43	−1.96
NLRC5-like	0	NOD-like receptor signaling	−5.35	295.84	2.82	1.02
Nuclear transcription factor Y subunit gamma	1.00E-100	NF-kappa B signaling	1.37	−2115.76	1.12	1.27
Caspase recruitment domain-containing protein 9	1.00E-167	NOD-like receptor signaling	0	1553.71	−9.94	1.02
CD4-like protein isoform 1 precursor	0	T cell receptor signaling	0	−1.02	−1.4	0
Interferon regulatory factor 3	0	Toll-like receptor	−1.37	297.15	1.23	1.13
CRFBx	0	Toll-like receptor	1.87	−153.97	2.41	1.34
Cytochrome P450 2M1	0	Cytochrome p450	−8.43	−3.47	−1.6	−2.85
UGT glucoronosyltransferase	0	Metabolism of xenobiotics	−5.9	−1.04	2.62	1.05
Glutathione s-transferase	0	Metabolism of xenobiotics	1.19	−1.04	−1.33	1.58
Proline serine-threonine phosphatase-interacting protein 2	0	Serine/Threonine phosphatases	−3.37	−1.18	3.75	1.08
Alpha 1 antiproteinase-like (antiprot1)	0	Serpins	−26.68	−7.73	−1.23	2.86
Antithrombin protein	0	Serpins	−9.73	1.02	−1.93	−1.39
Heat shock protein 47	0	Serpins	−1.11	−2.02	1.44	1.97
Tyrosine-protein kinase BTK putative	0	Tyrosine kinases	−5.06	1.13	2.62	−1.21
Serine/threonine/tyrosine-interacting protein	0	Tyrosine phosphatases	1.19	−8.32	1.12	2.11
Dual specificity protein phosphatase 12	0	Tyrosine phosphatases	1.19	2.4	4.12	−1.36
Epidermis-type lipoxygenase 3	0	Arachidonic acid metabolism	4.01	1	−27.62	1
Prostaglandin E synthase 3	5.00E-73	Arachidonic acid metabolism	−2.29	−2.11	1.1	2.43
Metabolism pathway genes						
Alcohol dehydrogenase 1-like	0	Glycolysis	−1.83	3.76	−1.75	1.43
Phosphoglycerate mutase 2-1	4.00E-179	Glycolysis	−1.47	−7.84	−1.04	2.85
Glyceraldehyde-3-phosphate dehydrogenase-2	0	Glycolysis	−1.55	−9.07	1.02	2.22
Beta-enolase	0	Glycolysis	−1.44	−6.77	−1.02	1.82
Fructose-bisphosphate aldolase A	1.00E-174	Glycolysis/PPP	−1.33	−9.66	−1.06	2.72
Suppressor of cytokine signaling 3	2.00E-147	Insulin signaling	1.83	2.25	−3.84	−3.22
Malate dehydrogenase 1	3.00E-180	TCA cycle	2.35	−1.34	−1.67	2.19

participates in several signaling processes such as MAPK signaling pathway. This gene is therefore directly related to the activation of cell proliferation processes and the regeneration of epithelial structures [36]. The observed up-regulation of this gene in the skin and head kidney of infested salmon suggests an activation of this process due to the wounds generated by the infestation process. The immunostimulatory effect seemed to reduce the expression of this gene. This indicates a protective effect since immunostimulants tend to decrease the parasitic load and, thus, the quantity and severity of skin wounds [28]. This expression pattern was also observed with the precursor of *plasminogen activator inhibitor 1*, which also plays an important role in blood vessels stabilization and regeneration, and in the *epidermis-type lipoxygenase 3* (*eLOX3*).

The lipoxygenases participate in arachidonic acid metabolism to produce leukotrienes that, together with other eicosanoids such as prostaglandins, are major pro-inflammatory compounds [37]. In particular, *eLOX3* in mammals is involved in epidermal barrier acquisition by modulating lipid metabolism and protein processing, in addition to activating peroxisome proliferator-activated receptors [38]. Thus, the up-regulation of this gene in salmon skin following *C. rogercresseyi* infestation might suggest a response to the need of integument repair, whereas the down-regulation observed in individuals under an immunostimulatory effect might be linked to acquired protection. The *prostaglandin E synthase 3* encodes for cytosolic synthase, and its down-regulation in the skin and head kidney after 15 days of sea lice infestation is in line with Skugor et al. [22], who found a down-regulation of *prostaglandin D synthase* in the skin of Atlantic salmon after 22 days of infestation with *L. salmonis*. There is evidence that in early sea lice infestation stages, the expressions of prostaglandin synthase genes increase in the skin [23,32] and kidney [22], but are down-regulated as exposure time increases, as was found in this study. This might be indicating a modulatory strategy induced by the parasite. On the other hand, the immunostimulatory effect seemed to up-regulate the gene even after 15 days of infestation, suggesting a possible increase on host-response potential to counteract parasitic strategies. Nevertheless, due to the complexity of eicosanoids metabolism and modes of action further research is necessary to better sustain the suggestions provided by this study.

The energy metabolism pathways for glycolysis, the pentose phosphate pathway, the TCA cycle, and gluconeogenesis were selected for inferences about the modulatory effects of the parasite and immunostimulants. These pathways were considered important due to their roles in ATP supply and, thus, in supplying the energy needed for responses to chronic deleterious conditions such as sea lice infestation. Genes involved in glycolysis were found to have significant roles in defining the effect of infestation and, in

general, were down-regulated in both tissues, suggesting a suppression of this pathway as inflicted by sea lice exposure. The chronic nature of this type of infestation most probably promotes the depletion of energy resources in salmon [39], thereby imposing a diversification of energy substrate metabolic pathways. The up-regulation of *Malate dehydrogenase 1*, a key enzyme in the TCA cycle and thus in ATP production, in the skin indicates that the cycle was activated and would be receiving substrate from other sources apart from glycolysis. The immunostimulatory effect of the diet again increased the expression of these same genes in the head kidney, which supports the premise that fish have higher resistance to sea lice infestation than unstimulated fishes. Genes of the glycolytic pathway down-regulated by infestation might also be related with the up-regulation of suppressors for the *cytokine signaling 3* or *SOCS3* genes. Proteins encoded by the SOCS family have insulin signaling inhibitory effects [40] and might be responsible for limiting the uptake of glucose and, consequently, the glycolytic pathway. On the other hand, the up-regulation of *SOCS3* also reveals part of parasite strategy for host immune evasion. In general, parasitic infestations induce SOCS gene expression, which contributes to infestation success [41]. Interestingly, the immunostimulant treatment shifted this tendency, and this gene was down-regulated, corroborating the overall protective effects previously observed. Nevertheless, some of the genes examined in the present study participate in more than one metabolic pathway, and further research is necessary to detail the pathways up- and down-regulated by infestation and dietary immunostimulation.

Given the cumulative impact of sea lice infestation on salmonid farms, dietary solutions promise to become interesting alternatives for reducing parasitic diseases. However, the host-parasite interaction system is complex, and it is difficult to develop specific immunostimulation solutions against *C. rogercresseyi*. In fact, previous work suggests that the divergent defense responses exhibited by different salmonid species to sea lice are indicative of a non-specific response to this pathogen [32]. In this scenario, the oral administration of immunostimulants could be the most cost effective and suitable treatment method for large aquaculture systems [26]. This method has been used in salmon production for nearly two decades, during which time there has been enhanced resistance to some current diseases [42]. Moreover, this strategy has not shown successful results in parasitic infestations affecting salmonid species, such as amoebic gill disease in *S. salar* [43]. A similar situation has been found in salmon species affected by sea lice, which, after orally-administered immunostimulation, showed increased levels of chemokines in the hosts, but the decrease in infestation levels did not reach even 50% of the parasitic load [44]. For these reasons, some researches have suggested the combined addition of antiparasitic chemicals and immunostimulants through oral administration, but with the disadvantage of using up to 3-fold the normal doses of delousing additives [45]. In other cases, no significant differences were found in parasitic load levels in groups treated with immunostimulants as compared to control groups [46].

In relation to *C. rogercresseyi* infestations, only one assessment exists in the public literature. In this, the successful reduction of infestation levels was achieved through the addition of poly-unsaturated aldehydes as an in-feed additive [47], but this implicated the use of a product with serious potential biosafety risks. In this context, there remains an urgent need to develop safe and effective immunostimulants. Transcriptomic data obtained from farmed salmon allow for associations to be made between the molecular responses of Atlantic salmon with the effects of natural in-feed additives, which the present data indicate as an appropriate complement to fish diets.

5. Conclusions

This study provides novel and important transcriptomic data that explains some defense mechanism activated/deactivated by the use of plant-derived in-feed additives in Atlantic salmon. Overall, the results suggest that the skin tissue better explains the defense mechanism of this salmon than does the head kidney. In-feed additives had a significant effect on infested salmons, triggering a different defense response parasite infestation than infested individuals fed a control diet. These results suggest that the evaluated in-feed additives, extracted from plants, have a positive effect in laboratory conditions, and could therefore be used as an immunomodulator in field trials. Overall, this study provides important information for the integrated management of parasitic diseases in salmon farms.

Acknowledgements

This study was co-funded by FONDAP (15110027), FONDECYT (1150077) and CONICYT-PCHA/Doctorado Nacional/2015-21151044 grants from CONICYT-Chile and EWOS Innovation, Chile.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.09.009.

References

[1] E.J. Niklitschek, D. Soto, A. Lafon, C. Molinet, P. Toledo, Southward expansion of the Chilean salmon industry in the Patagonian Fjords: main environmental challenges, Rev. Aquac. 5 (2013) 172–195.
[2] B. Bustos-Gallardo, The ISA crisis in Los Lagos Chile: a failure of neoliberal environmental governance? Geoforum 48 (2013) 196–206.
[3] O. Torrissen, S. Jones, F. Asche, A. Guttormsen, O.T. Skilbrei, F. Nilsen, et al., Salmon lice – impact on wild salmonids and salmon aquaculture, J. Fish Dis. 36 (2013) 171–194.
[4] M.J. Costello, The global economic cost of sea lice to the salmonid farming industry, J. Fish Dis. 32 (2009) 115–118.
[5] L. Burridge, J.S. Weis, F. Cabello, J. Pizarro, K. Bostick, Chemical use in salmon aquaculture: a review of current practices and possible environmental effects, Aquaculture 306 (2010) 7–23.
[6] C.M. Couillard, L.E. Burridge, Sublethal exposure to azamethiphos causes neurotoxicity, altered energy allocation and high mortality during simulated live transport in American lobster, Ecotoxicol. Environ. Saf. (2014) 1–9.
[7] K.H. Langford, S. Øxnevad, M. Schøyen, K.V. Thomas, Do antiparasitic medicines used in aquaculture pose a risk to the Norwegian aquatic environment? Environ. Sci. Technol. 48 (2014) 7774–7780.
[8] O.B. Samuelsen, B.T. Lunestad, E. Farestveit, E.S. Grefsrud, R. Hannisdal, B. Holmelid, et al., Mortality and deformities in European lobster (*Homarus gammarus*) juveniles exposed to the anti-parasitic drug teflubenzuron, Aquat. Toxicol. (Amsterdam, Netherlands) 149 (2014) 8–15.
[9] O.B. Samuelsen, B.T. Lunestad, R. Hannisdal, R. Bannister, S. Olsen, T. Tjensvoll, et al., Distribution and persistence of the anti sea-lice drug teflubenzuron in wild fauna and sediments around a salmon farm, following a standard treatment, Sci. Total Environ. 508 (2015) 115–121.
[10] J.L. Van Geest, L.E. Burridge, Ka Kidd, Toxicity of two pyrethroid-based anti-sea lice pesticides, AlphaMax® and Excis®, to a marine amphipod in aqueous and sediment exposures, Aquaculture 434 (2014) 233–240.
[11] J.P. Benskin, M.G. Ikonomou, B.D. Surridge, C. Dubetz, E. Klaassen, Biodegradation potential of aquaculture chemotherapeutants in marine sediments, Aquac. Res. 2014 (2014) 1–16.
[12] M.D. Fast, Fish immune responses to parasitic copepod (namely sea lice) infection, Dev. Comp. Immunol. 43 (2014) 300–312.
[13] S.C. Johnson, L.J. Albright, P.B. Station, Comparative susceptibility and histopathology of the response of naive Atlantic, chinook and coho salmon to experimental infection with *Lepeophtheirus salmonis* (Copepoda: Caligidae), Dis. Aquat. Org. 14 (1992) 179–193.
[14] M.W. Jones, C. Sommerville, J. Bron, The histopathology associated with the juvenile stages of *Lepeophtheirus salmonis* on the Atlantic salmon, *Salmo salar* L. J. Fish Dis. 13 (1990) 303–310.
[15] H. Jónsdóttir, J.E. Bron, R. Wootten, J.F. Turnbull, The histopathology associated with the pre-adult and adult stages of *Lepeophtheirus salmonis* on the Atlantic salmon, *Salmo salar* L. J. Fish Dis. 15 (1992) 521–527.
[16] D.T. Nolan, P. Reilly, S.E.W. Bonga, Infection with low numbers of the sea louse *Lepeophtheirus salmonis* induces stress-related effects in postsmolt Atlantic

salmon (*Salmo salar*), Can. J. Fish. Aquat. Sci. 56 (1999) 947–959.
[17] B.J. Sutherland, K.W. Koczka, M. Yasuike, S.G. Jantzen, R. Yazawa, B.F. Koop, et al., Comparative transcriptomics of Atlantic *Salmo salar*, chum *Oncorhynchus keta* and pink salmon *O. gorbuscha* during infections with salmon lice *Lepeophtheirus salmonis*, BMC Genom. 15 (2014) 1–17.
[18] G.N. Wagner, M.D. Fast, S.C. Johnson, Physiology and immunology of *Lepeophtheirus salmonis* infections of salmonids, Trends Parasitol. 24 (2008) 176–183.
[19] A. Wells, C.E. Grierson, M. MacKenzie, I.J. Russon, H. Reinardy, C. Middlemiss, et al., Physiological effects of simultaneous, abrupt seawater entry and sea lice (*Lepeophtheirus salmonis*) infestation of wild, sea-run brown trout (*Salmo trutta*) smolts, Can. J. Fish. Aquat. Sci. 63 (2006) 2809–2821.
[20] J.M. Bowers, A. Mustafa, D.J. Speare, G.A. Conboy, M. Brimacombe, D.E. Sims, et al., The physiological response of Atlantic salmon, *Salmo salar* L., to a single experimental challenge with sea lice, *Lepeophtheirus salmonis*, J. Fish Dis. 23 (2000) 165–172.
[21] M.P. González, S. Marín, L. Vargas-Chacoff, Effects of *Caligus rogercresseyi* (Boxshall & Bravo 2000) infestation on physiological response of host *Salmo salar* (Linnaeus 1758): establishing physiological thresholds, Aquaculture 438 (2015) 47–54.
[22] S. Skugor, K.A. Glover, F. Nilsen, A. Krasnov, Local and systemic gene expression responses of Atlantic salmon (*Salmo salar L.*) to infection with the salmon louse (*Lepeophtheirus salmonis*), BMC Genom. 9 (2008) 498.
[23] T.M. Tadiso, A. Krasnov, S. Skugor, S. Afanasyev, I. Hordvik, F. Nilsen, Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed biphasic responses coinciding with the copepod-chalimus transition, BMC Genom. 12 (2011) 1–17.
[24] H. Holm, N. Santi, S. Kjøglum, N. Perisic, S. Skugor, Ø. Evensen, Difference in skin immune responses to infection with salmon louse (*Lepeophtheirus salmonis*) in Atlantic salmon (*Salmo salar L.*) of families selected for resistance and susceptibility, Fish Shellfish Immunol. 42 (2015) 384–394.
[25] E. Ringø, R.E. Olsen, I. Jensen, J. Romero, H.L. Lauzon, Application of vaccines and dietary supplements in aquaculture: possibilities and challenges, Rev. Fish Biol. Fish. 24 (2014) 1005–1032.
[26] D. Barman, P. Nen, S.C. Mandal, V. Kumar, Immunostimulants for Aquaculture Health Management, Mar. Sci. Res. Dev. 3 (2013) 1–11.
[27] L.B. Jensen, F. Provan, E. Larssen, J.E. Bron, A. Obach, Reducing sea lice (*Lepeophtheirus salmonis*) infestation of farmed Atlantic salmon (*Salmo salar* L.) through functional feeds, Aquac. Nutr. (2014) 1–11.
[28] M. Sakai, Current research status of fish immunostimulants, Aquaculture 172 (1999) 63–92.
[29] N. Van Hai, The use of medicinal plants as immunostimulants in aquaculture: a review, Aquaculture 446 (2015) 88–96.
[30] M.P. González, S.L. Marín, L. Vargas-Chacoff, Effects of *Caligus rogercresseyi* (Boxshall and Bravo, 2000) infestation on physiological response of host Salmo salar (Linnaeus 1758): establishing physiological thresholds, Aquaculture 438 (2015) 47–54.
[31] A.K. Chakravarthy, New horizons in insect science: toward sustainable pest management, 2015, pp. 239–257.
[32] L.M. Braden, D.E. Barker, B.F. Koop, S.R.M. Jones, Comparative defense-associated responses in salmon skin elicited by the ectoparasite *Lepeophtheirus salmonis*, Comp. Biochem. Physiol. Part D Genomics Proteomics 7 (2012) 100–109.
[33] S.C. Johnson, S. Bravo, K. Nagasawa, Z. Kabata, J. Hwang, J. Ho, et al., A review of the impact of parasitic copepods on marine aquaculture, Zool. Stud. 43 (2004) 229–243.
[34] S. Bravo, The reproductive output of sea lice *Caligus rogercresseyi* under controlled conditions, Exp. Parasitol. 125 (2010) 51–54.
[35] L.M. Braden, B.F. Koop, S.R.M. Jones, Signatures of resistance to *Lepeophtheirus salmonis* include a T H 2-type response at the louse-salmon interface, Dev. Comp. Immunol. 48 (2015) 178–191.
[36] A. Tarnawski, I.L. Szabo, S.S. Husain, B. Soreghan, Regeneration of gastric mucosa during ulcer healing is triggered by growth factors and signal transduction pathways, J. Physiol. Paris 95 (2001) 337–344.
[37] A. Daugschies, A. Joachim, Eicosanoids in parasites and parasitic infections, Adv. Parasitol. 46 (2000) 181–240.
[38] N. Epp, G. Fürstenberger, K. Müller, S. de Juanes, M. Leitges, I. Hausser, et al., 12R-lipoxygenase deficiency disrupts epidermal barrier function, J. Cell Biol. 177 (2007) 173–182.
[39] S.W. Bonga, The stress response in fish, Physiol. Rev. 77 (1997) 591–625.
[40] P. Lebrun, E. Van Obberghen, SOCS proteins causing trouble in insulin action, Acta Physiol. 192 (2008) 29–36.
[41] T. Wang, B. Gorgoglione, T. Maehr, J.W. Holland, J.L.G. Vecino, S. Wadsworth, et al., Fish suppressors of cytokine signaling (SOCS): gene discovery, modulation of expression and function, J. Signal Transduct. (2011) 2011.
[42] B. Robertsen, G. RΘRstad, R. Engstad, J. Raa, Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls, J. Fish Dis. 13 (1990) 391–400.
[43] D. Zilberg, V.L. Findlay, P. Girling, B.L. Munday, Effects of treatment with levamisole and glucans on mortality rates in Atlantic salmon (*Salmo salar* L.) suffering from amoebic gill disease, Bull. Eur. Assoc. Fish Pathol. 20 (2000) 23–27.
[44] J.M. Covello, S.E. Friend, S.L. Purcell, J.F. Burka, R.J.F. Markham, A.W. Donkin, et al., Effects of orally administered immunostimulants on inflammatory gene expression and sea lice (*Lepeophtheirus salmonis*) burdens on Atlantic salmon (*Salmo salar*), Aquaculture 366 (2012) 9–16.
[45] J. Poley, S. Purcell, O. Igboeli, A. Donkin, H. Wotton, M. Fast, Combinatorial effects of administration of immunostimulatory compounds in feed and follow-up administration of triple-dose SLICE® (emamectin benzoate) on Atlantic salmon, *Salmo salar* L., infection with *Lepeophtheirus salmonis*, J. Fish Dis. 36 (2013) 299–309.
[46] F. Provan, L.B. Jensen, K.E. Uleberg, E. Larssen, T. Rajalahti, J. Mullins, et al., Proteomic analysis of epidermal mucus from sea lice-infected Atlantic salmon, *Salmo salar* L. J. Fish Dis. 36 (2013) 311–321.
[47] J. Troncoso, J. Gonzalez, J. Pino, K. Ruohonen, A. El Mowafi, J. Gonzalez, et al., Effect of polyunsatured aldehyde (A3) as an antiparasitary ingredient of *Caligus rogercresseyi* in the feed of Atlantic salmon, *Salmo salar*, Lat. Am. J. Aquat. Res. 39 (2011) 439–448.