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The impact of *Piscirickettsia salmonis* infection on genome-wide DNA methylation profile in Atlantic Salmon

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ABSTRACT

Salmon rickettsial septicaemia (SRS), caused by the bacteria *Piscirickettsia salmonis (P. salmonis),* is responsible for significant mortality in farmed Atlantic salmon in Chile. Currently there are no effective treatments or preventive measures for this disease, although genetic selection or genome engineering to increase salmon resistance to SRS are promising strategies. The accuracy and efficiency of these strategies are usually influenced by the available biological background knowledge of the disease. The aim of this study was to investigate DNA methylation changes in response to *P. salmonis* infection in the head kidney and liver tissue of Atlantic salmon, and the interaction between gene expression and DNA methylation in the same tissues. The head kidney and liver methylomes of 66 juvenile salmon were profiled using reduced representation bisulphite sequencing (RRBS), and compared between *P. salmonis* infected animals (3 and 9 days post infection) and uninfected controls, and between SRS resistant and susceptible fish. Methylation was correlated with matching RNA-Seq data from the same animals, revealing that methylation in the first exon leads to an important repression of gene expression. Head kidney methylation showed a clear response to the infection, associated with immunological processes such as actin cytoskeleton regulation, phagocytosis, endocytosis and pathogen associated pattern receptor signaling. Our results contribute to the growing understanding of the role of methylation in regulation of gene expression and response to infectious diseases and could inform the incorporation of epigenetic markers into genomic selection for disease resistant and the design of diagnostic epigenetic markers to better manage fish health in salmon aquaculture.

1. Introduction

DNA methylation is a fundamental epigenetic mechanism that involves the conversion of cytosine (predominantly in the CG or CpG dinucleotides) to 5′-methylcytosine by DNA methyltransferases (DNMTs) such as *DNMT3A* and *DNMT3B* [1]. DNA methylation plays a significant role in maintaining the stability of the genome by silencing transcription of repetitive elements in the genome [1]. While DNMTs promote methylation, Ten-Eleven Translocation (TET) methylcytosine dioxygenases including *TET1*, *TET2* and *TET3* promote demethylation of the methylated cytosines [2,3]. It is the interplay of DNMT and TETs (i.e. methylation and demethylation) that transcends into coordinated transcriptomic changes to ensure appropriate cellular responses to internal or external stimuli such as pathogen infection [4,5]. Together with other epigenetic mechanisms, DNA methylation is involved in the regulation of gene expression and thus in the modulation of most biological processes [6–8]. Increased methylation levels in the promoter regions of genes has been associated with repressed gene expression, however positive associations between DNA methylation and gene expression have also been reported [5]. Methylation changes in the 1st exon and 1st intron have also shown a strong antagonistic association with gene expression [9–11], indicating that DNA methylation influence on gene expression is not restricted to the promoter regions. In addition, methylation profiles are greatly tissue and cell type specific, which is linked to ensuring cell type and tissue differentiation, growth function specialization in the body [2,4,6].

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Pathogen infection can trigger DNA methylation changes that may lead to changes in gene expression in the host. These changes can be part of the host regulatory mechanisms leading to immune activation [5,6,12]. Nevertheless, pathogens have also developed mechanisms of manipulating their host's methylomes to enable their survival and multiplication within the host cells [5,13–15]. Global and targeted DNA methylation changes upon infection by different bacterial species such as *Mycobacteria tuberculosis* [15–17], *Escherichia coli* [18,19] and *Salmonella* spp. [20,21] has been demonstrated in different species. DNA methylations patterns can be inherited [22], hence their contribution to genetically to phenotypic variability between individuals. With respect infection response, host methylation profiles in response to a pathogen infection can vary between individuals with divergent susceptibility to the pathogen, for example, Xui et al. previously reported differences in DNA methylation in the kidney, liver and spleen between resistant and susceptible Chinese tongue sole during *Vibrio harveyi* infection [22]. The identification of methylation signatures associated with pathogen infection and physiological or phenotypic changes upon infection could aid in the development of targeted specific therapeutic drugs [5,6], diagnostic/prognostic epigenomic biomarkers [5,23] and vaccines [24]. In livestock and aquaculture species, the identification of DNA methylation signatures related with genetic variation in host resistance can enable the identification of functional and regulatory genomic features that could be integrated into genomic selection to improve the accuracy of selection for disease resistance [25,26].

Piscirickettsia salmonis (*P. salmonis*) is the cause of salmon rickettsial septicaemia (SRS) and a major threat to farmed salmonids. *P. salmonis* is a gram-negative facultative intracellular bacteria that mainly invades and multiplies in macrophage cells [27]. SRS has been reported in numerous farmed salmon producing countries including Chile, Norway, United Kingdom, Canada, and the United States [28]. However, *P. salmonis* is currently only a major threat in Chile, where this disease is endemic and represents one of the biggest problems faced by the salmon aquaculture industry. SRS affects the seawater stage of the production cycle, causing over 50% of the mortalities related to infectious diseases [29], with annual loses of US$300 M – US$700 M [30–32]. To date, several strategies have been applied to prevent or control the disease, including vaccination, reduced stocking densities, antimicrobial treatments and increased biosecurity at farms [32,33]. Nonetheless, SRS continues to be a major problem [31–33], causing an excessive use of antimicrobials that can promote the evolution of antimicrobial resistance in bacterial communities [28,31,32,34]. Genetic improvement of host resistance is a promising and complementary prevention strategy, and additive genetic variation for resistance to SRS has been detected in various farmed populations, yielding heritability estimates ranging from 0.11 to 0.43 [35–37]. In fact, genetic improvement for SRS resistance is already being implemented via genomic selection in different commercial breeding programs for Atlantic salmon in Chile [38–40].

The head kidney and the liver are key immune organs in teleost fish. The head kidney is a primary lymphoid organ with hemopoietic activity, key in the generation and maturation of B and T lymphocyte cells and a major source of phagocytic macrophages [41]. On the other hand, the liver is a fundamental part of the complement system and also plays a major role in the phagocytosis of invading pathogen via Kuppfer cells [41–43]. Previous efforts to investigate responses to *P. salmonis* have been focused mainly in these two tissues and spleen [36,44–47]. Although the pathogenesis of SRS has not yet been fully characterized, histological lesions have been observed in the liver during *P. salmonis* infections [48]. Epigenetic changes, such as DNA methylation, may partly the transcriptional changes observed in response to *P. salmonis* infection, and consequently contribute to molecular and phenotypic variation during SRS. In this line, a study of coho salmon (*Oncorhynchus kisutch*) response to SRS highlighted variation in the methylation profile of the spleen during infection, and linked this variation to various immune pathways [49].

The role of DNA methylation in response to SRS in Atlantic salmon has not been studied. In this study, methylation was studied in two Atlantic salmon lymphoid tissues (head kidney and liver) following experimental infection with *P. salmonis*, and integrated with matching transcriptomic data from the same animals. Differences in methylation and their impact on gene expression were examined between infected fish and controls, and between fish classified as either SRS resistant or susceptible based on genomic breeding values.

2. Materials and methods

2.1. Animals and SRS challenge trial

A total of 2265 Atlantic salmon pre-smolts (average weight 135 ± 47 g) from 96 full sibling families from the breeding population of AquaInnovo (Salmones Chaicas, Xth Region, Chile) were utilized in the current study. Through the course of the experiment, these fish were kept in 3 tanks of 7 m^3 volume each, with an even number of animals from each family in each tank. Prior to the challenge experiment, all fish used in the study were vaccinated against *Flavobacterium*, infectious pancreatic necrosis virus (IPNV; Alpha Ject Flavo + IPN) and infectious salmon anaemia virus (ISAV; Alpha Ject Micro 1-ISA). Three weeks post vaccination, fish were screened for ISAV, IPNV, *Renibacterium salmoninarum*, *Flavobacterium psycrophilum* and Mycoplasma using qPCR, and for bacterial contamination using culture in TSA, TSA + salt, and *Piscirickettsia salmonis* agar at 18 °C and 35 °C at Laboratorio Antares S.A. (Puerto Montt, Chile). Prior to the disease challenge, 48 fish (uninfected) were sacrificed as the control group, with head kidney and liver sampled for transcriptomic and methylation assays. The remaining fish were intraperitoneally injected with 0.2 mL of a 1/2030 dilution of live *P. salmonis* bacteria. This dose of the inoculum corresponds to the median lethal dose (LD50), previously estimated with the Reed-Muench method using a group of 300 fish from the same families challenged with 1/10, 1/100, 1/1000 and 1/10000 dilutions of the bacteria. Therefore, a mortality rate of approximately 50% was expected. The inoculated fish were maintained in the 3 tanks (~755 animals per tank) under the following conditions: temperature 14 ± 0.0 °C, salinity 30.4 ± 0.5%, pH 7.4 ± 0.1, and oxygen saturation 102.2 ± 6.0%. At 3 days and 9 days post infection 48 animals from the same tank were euthanized and liver and head kidney were sampled for RNA sequencing and methylation assays. The experiment was terminated after 47 days when mortality plateaued. The overall mortality rate of the challenged fish was 34.07 ± 0.02%. All live fish used in this study – controls (n = 48), 3 dpi (n = 48), 9 dpi (n = 48) and fish surviving at the end of the challenge (n = 1509) – were anesthetized and euthanized by placing them in 30 mg/L benzocaine solution for about 20 min. All animals in the experiment were genotyped using a 968 SNP panel that was then imputed to ~46 K SNP genotypes as described by Moraleda et al. 2021. Using the genomic best linear unbiased prediction model (GBLUP), a mixed linear animal model implemented by ASReml software [50], these imputed genotypes were then utilized to predict the estimated genomic breeding values (GEBVs) for resistance to SRS (measured as mortality / survival) for each animal (detailed methods in Moraleda et al. 2021). The GEBVs were then used to classify the animals in resistant and susceptible groups.

The experimental immune challenge of the fish was performed according to the local and national regulatory systems and were approved by the Animal Bioethics Committee (ABC) of the Faculty of Veterinary and Animal Sciences of the University of Chile (Santiago, Chile), Certificate N° 01–2016, which based its decision on the Council for International Organizations of Medical Sciences (CIOMS) standards, in accordance with the Chilean standard NCh-324-2011.

2.2. Tissue collection

During the trial a subset of animals were sampled for transcriptomic (RNA-Seq) and methylation (Reduced Representation Bisulphite Sequencing, RRBS) analyses. All sampled animals were ethically

anesthetized and euthanized before collecting the samples. In total, liver and head kidney tissue samples were collected from 144 animals at three time points: 48 unchallenged (control) fish, 48 pathogen-challenged fish at 3 days post infection (dpi), and 48 pathogen-challenged fish at 9 dpi. Tissues dissected from each animal were individually stored in RNAlater at 4 °C for 24 h, and thereafter kept at -20 °C until DNA extraction, and RNA extraction as previously described by Moraleda et al. 2021.

2.3. RRBS library preparation and sequencing

A total of 66 samples (i.e. 33 samples of each tissue) including 7 x control, 13 x 3dpi, and 13 x 9dpi samples were selected for the RRBS analyses based on the availability of RNA sequencing data (from Moraleda et al. 2021), their EBVs for resistance to SRS, and the availability of high quality DNA for bisulfite conversion and sequencing. Bisulfite converted reduced representation genomic DNA libraries were prepared using the Diagenode Premium RRBS kit [51] following the manufacturer's instructions. Briefly, 100 ng of genomic DNA from each of the 66 samples was digested with the restriction enzyme *Msp*I for 12 h, followed by fragment end-repair, A-tailing, and adapter ligation. Methylated and unmethylated spike-in controls were added to monitor bisulfite conversion efficiency. Individual libraries were quantified in duplicate by qPCR. Samples with similar qPCR threshold cycle (Ct) values were multiplexed in equimolar amounts in pools of six. Pools were then subjected to bisulfite conversion. Thereafter, RRBS libraries were enriched by PCR and purified with AMPure® XP beads (#A63881, Beckman Coulter). Quality assessment of the RRBS libraries was performed by verifying the fragment size distribution on an Agilent 2200 Bioanalyzer (Agilent Technologies). Libraries were quantified using a high sensitivity assay on a Qubit 3.0 Fluorometer (Life Technologies, Thermo Fisher Scientific), and then pooled at equimolar concentrations for sequencing on three flow cell lanes of an Illumina NovaSeq S1 platform (50 bp paired-end sequencing) at Edinburgh Genomics (University of Edinburgh).

2.4. RRBS data processing and methylation profiling

Raw sequence read data quality was initially evaluated using FastQC software Version 0.11 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw sequence data were then cleaned by removing low quality base calls (Phred score < 20) from the ends of the reads, short reads (< 20 bp) and Illumina sequencing adapters using Trim Galore Version 0.5.0 software [52] with default RRBS paired-end parameters. Methylation profiling from the cleaned sequence data was performed using Bismark Version 0.22.3 pipeline tools [53]. To facilitate bisulphite alignment, the Atlantic salmon reference genome (GCF_000233375.1_ICSASG_v2 downloaded from NCBI on 19/04/2020) was bisulphite converted in silico to C- > T (forward) and G- > A (reverse) versions using the bismark_genome_preparation script part of Bismark Version 0.22.3 pipeline (https://github.com/FelixKrueger/Bismark/archive/0.22.3.tar.gz). The bisulphite-converted clean RRBS sequence reads were then aligned to the *in-silico* bisulphite-converted reference genome version using the Bismark script (part of Bismark v0.22.3 pipeline) that utilizes bowtie2 [54] as the underlying short read aligner. Subsequently, the methylation state of each cytosine in the genome was called from the alignments using the same Bismark script (part of Bismark v0.22.3 pipeline). The methylation call for all profiled cytosine (C) nucleotides were extracted from the alignment bam files using the bismark_methylation_extractor script (part of Bismark v0.22.3 pipeline) into CpG methylation coverage (count) files that were used for downstream analyses. Due to the inability of RRBS to distinguish between C/T single nucleotide polymorphisms (SNPs) and true C-T bisulphite conversion [55], all C/T SNPs were filtered out from profiled CpG sites based on previous whole genome sequencing data from the same population as described by Robledo et al. [56]. Briefly, this whole genome SNP genotype data were called from high quality Illumina whole genome paired-end (150 bp) sequence data of 100 fish belonging to the same families as the fish used in the current study. A SNP was considered reliable with a coverage of ≥10 aligned reads. The R package Circulize Version 0.4.10 [57] was used to visualize genome-wide basal (unchallenged animals) methylation patterns and differences in methylation between liver and head kidney samples.

2.5. Genomic annotation of CpG sites and functional enrichment analyses

The identified CpG sites were functionally annotated according to the genomic features in the salmon genome annotation file (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/233/375/GCF_000233375.1_ICSASG_v2/GCF_000233375.1_ICSASG_v2_genomic.gtf.gz, downloaded on 19/04/2020) using the annotatePeaks.pl tool from the HOMER software (http://homer.ucsd.edu/homer/ngs/annotation.html). CpG sites were annotated as located either in the putative proximal promoters / transcription start site (TSS) regions (defined as -1kbp to +100 bp around the TSS), exons, introns, the transcription termination site (TTS) regions (defined as -100 bp to +1kbp around the TTS) or in intergenic regions.

2.6. Differential methylation analyses

Differential methylation analysis was performed using a Bioconductor R package edgeR Version 3.28.1 [58]. Firstly, CpG sites which had low coverage (< 10 reads per sample), those that were either always methylated or unmethylated across all samples, and those located on unplaced-scaffolds without annotated genes were removed from the analysis. Principal component analysis was performed on the methylation proportion with the *prcomp* function implemented in R to visualize the distribution of the samples according to their overall methylation patterns, and outlier samples notably isolated from the major clusters were discarded which included three liver samples (one control; one 3 dpi and one 9 dpi) and two head kidney samples (one 3 dpi and one 9 dpi). We retained 6 control, 12 × 3 dpi and 12 × 9 dpi samples for differential methylation analysis for the liver, and 7 control, 11 × 3 dpi and 9 × 9 dpi samples were retained for analysis in the head kidney. The read counts of each CpG site within a sample library were then normalized by scaling both methylated and unmethylated counts to the average library size of the sample (i.e., average of methylated and unmethylated libraries). A negative binomial generalized linear model implemented in edgeR [58] was used to model the normalized counts between the healthy (control) and infected (3 dpi and 9 dpi) animals with sex included as a fixed effect in the model. Differential methylation between groups was tested via likelihood ratio tests (LRT), and a CpG site was considered significantly differentially methylated with a false discovery rate (Benjamini – Hochberg correction) < 0.1. Heatmaps were plotted to visualize hierarchical clustering of the samples based on the differentially methylated CpG sites using *heatmap.2* function of the R package gplots v3.1.1 [59]. CpG sites located in or in close proximity to genes according to the criteria described above were used for functional pathway enrichment analyses. Pathway enrichment analysis was performed using the KEGG Orthology-Based Annotation System (KOBAS) Version 3.0.3 [60], with pathways showing a p-value <0.05 considered significantly enriched. Visualization of interactions between differentially methylated genes and KEGG pathways was performed using Cytoscape Version 3.9.1 [61].

2.7. Integrating DNA methylation and transcriptome expression

Gene expression data for the 33 liver and 28 head kidney samples used in the current study were obtained from our previous study, Moraleda et al. [36]. Briefly, total RNA was extracted from each sample using the TRI Reagent RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA). Extracted RNA was then cleaned and purified using the Qiagen RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). Subsequently,

cDNA libraries were prepared using the llumina TruSeq RNA Sample-Prep Kit v2 (Illumina, San Diego, CA), and paired-end sequenced on the Illumina Hiseq 4000 platform with read length of 75 bases. Raw sequence data was cleaned of adaptors and low-quality sequences using the Trimmomatic software [62]. Subsequently, gene transcript abundances were quantified through pseudoalignment of clean reads to the Atlantic salmon reference transcriptome using the Kallisto pseudo-aligner [63]. To normalize count data, counts per million (cpm) for each sample were calculated using the Bioconductor R package edgeR [58]. For each tissue, Pearson's correlations between percentage of DNA methylation of the CpG sites and cpm of their overlapping genes were computed using the R package Hmisc [64], and a correlation was considered significant at a *p*-value <0.05. Pearson's correlations between average (across samples) transcript expression and average DNA methylation for the different genomic features (i.e., putative proximal promoter-TSS, introns, exons, TTS, and intergenic regions as defined above) were also computed.

3. Results

3.1. RRBS sequencing and DNA methylation summary statistics

Sequencing of the liver and head kidney RRBS libraries yielded on average 40.1 ± 2.2 and 41.2 ± 1.87 million (mean ± SD) raw paired-end reads after quality control, respectively. There were no significant differences between head kidney and liver samples (Fig. S1A in Supplementary file 1). An average of 42.5% of the reads aligned to a unique genomic position in the Atlantic salmon genome assembly (Fig. S1B in Supplementary file 1). Most of the methylated cytosines identified in the sequenced libraries were within CpG sites (>84%; Fig. S1B and Fig. S1C in Supplementary file 1), and on average 3.6 ± 0.1 million and 3.8 ± 0.05 million CpG sites were profiled per sample in the liver and head kidney, respectively (Fig. S1D in Supplementary file 1). The methylation levels of 693,215 and 961,595 CpG sites were profiled in all liver and head kidney samples respectively.

3.2. Atlantic salmon head kidney and liver methylation patterns in unchallenged fish

A total of 308,198 and 247,252 CpG sites with sufficient sequencing coverage (≥10 read counts) were identified as showing variation in methylation levels in the head kidney and liver control samples, respectively. Each tissue had a markedly different methylation signature which allows the discrimination of each sample based on their tissue of origin (Fig. 1A). A total of 10,474 sites across the genome were significantly (FDR < 0.1) differentially methylated between the two tissues (6456 showed higher methylation in the head kidney and 4018 in the liver) (Fig. 1A). These differences are relatively small considering that over 200,000 variable CpG sites were detected in each tissue. Nonetheless, certain sites were fully unmethylated across all head kidney (8719 sites) and liver (10,743 sites) samples, and these showed a different genomic distribution between the two tissues (Fig. 1A), possibly connected to the role of methylation in tissue differentiation and function. In both liver and head kidney, fully methylated and variably methylated CpGs were predominantly located in intergenic and intronic regions (>85% of all methylated sites), while fully unmethylated sites were predominantly located in putative proximal promoter-TSS and exonic regions (>60% of all unmethylated sites) (Fig. 1B). CpG sites showing variation in methylation levels within each tissue were mainly located in the intergenic (49.9 ± 0.94%) and intronic (39.0

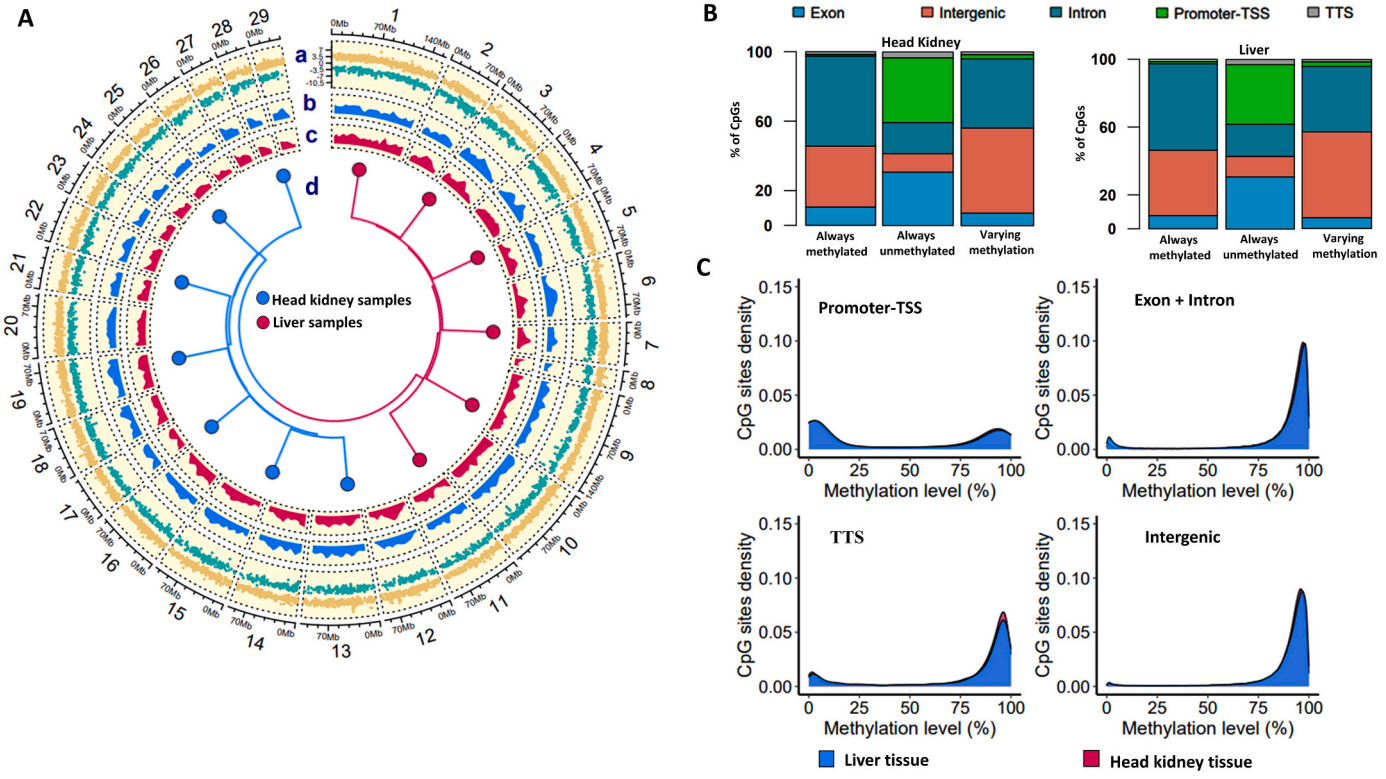

Fig. 1. General methylation patterns in head kidney and liver tissue of unchallenged animals. A) Circos plot showing differential methylation between head kidney and liver samples (Track: a), Genomic distribution of always fully unmethylated CpG sites in the head kidney tissue (Track: b), Genomic distribution of always fully unmethylated CpG sites in the liver tissue (Track: c), Dendrogram plot showing unchallenged fish head kidney and liver samples clustering based on their methylation profiles (Track: d). B) Distribution of the three classes of methylation (i.e., fully methylated in all samples, fully unmethylated in all samples and varying methylation between samples) in the different genomic features. C) Methylation variability within the different genomic features for the head kidney and liver tissues.

± 0.49%) regions (Fig. 1B). Similarly, intragenic regions (exons and introns), TTS, and intergenic regions were heavily skewed towards high (>75%) methylation (84.6 ± 2.45% of the CpGs), while in putative proximal promoter-TSS regions there was a higher proportion (39.3 ± 1.28% of the CpGs) of low (<25%) methylation levels (Fig. 1C). This might suggest different functions for methylation depending on the genomic region, with promoter-TSS regions showing a more variable pattern due to their more direct role controlling gene expression. Infection with *P. salmonis* did not significantly modify the distribution of methylation patterns described above (Fig. S2 in Supplementary file 1).

3.3. Integrating gene expression and DNA methylation

The correlation between methylation levels and gene expression was assessed to understand the potential impact of methylation on transcriptional regulation. Out of 126,625 and 100,891 CpG sites showing variable methylation in the head kidney and liver, respectively, only 2310 and 1306 showed significant (p-value <0.05) correlation with the expression of neighboring genes (Supplementary file 2). The highest correlations were > |0.7|, but most of the values ranged between |0.4| and |0.5| (Fig. 2A and B). Approximately 60% of the methylated sites showed a negative correlation with gene expression, while 40% showed positive correlation (Supplementary file 2). Overall, methylation in the putative proximal promoter-TSS regions, the 1st exon, the TTS region, and to a lesser extent the first intron, showed the strongest negative correlations with gene expression (Fig. 2C and D).

3.4. SRS-induced changes in head kidney and liver methylation patterns

Comparison of the methylomes of *P. salmonis*-infected and healthy animals revealed a higher number of sites differentially methylated in head kidney than in liver (Fig. 3A). In head kidney, 965 differentially methylated CpG sites (DMCpGs) were identified between control and 3 dpi challenged animals (644 sites increased methylation and 321 decreased in infected samples), and 704 between control and 9 dpi infected samples (328 increased methylation and 376 decreased methylation) (Fig. 3A, Supplementary file 3). In contrast, the liver just showed 10 DMCpGs between controls and 3 dpi samples, and 53 DMCpGs between controls and 9 dpi samples (Fig. 3A, Supplementary file 3). This contrasts with the findings of our previous transcriptomic

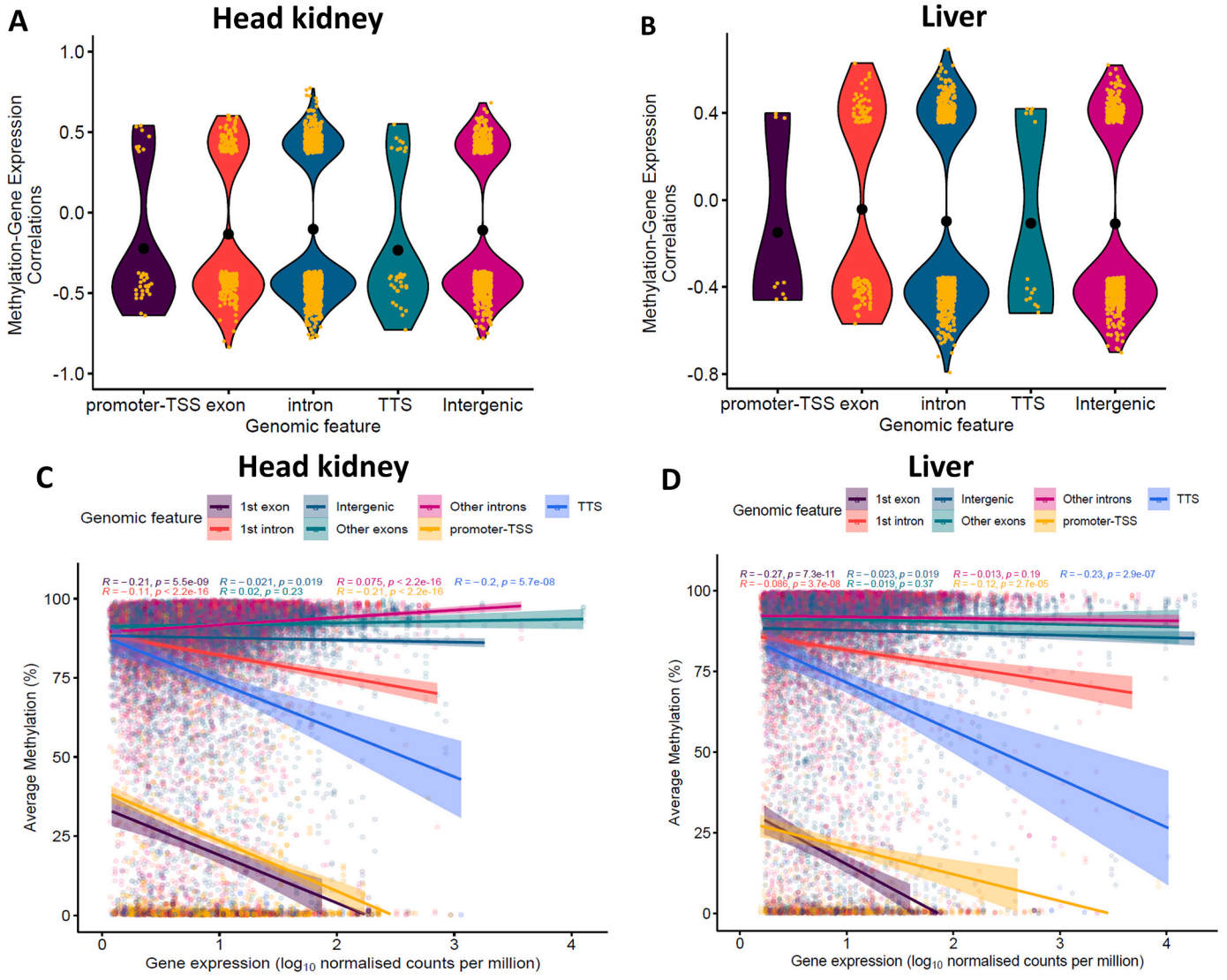

Fig. 2. Global correlation between methylation and gene expression; A) Violin plot showing the distribution of the correlations between DNA methylation and gene expression in head kidney, B) Violin plot showing the distribution of the correlations between DNA methylation and gene expression in liver, C) correlation plot showing the correlation between average gene expression and average methylation for each different genomic feature in the head kidney, D) correlation plot showing the correlation between average gene expression and average methylation for each different genomic feature in the liver.

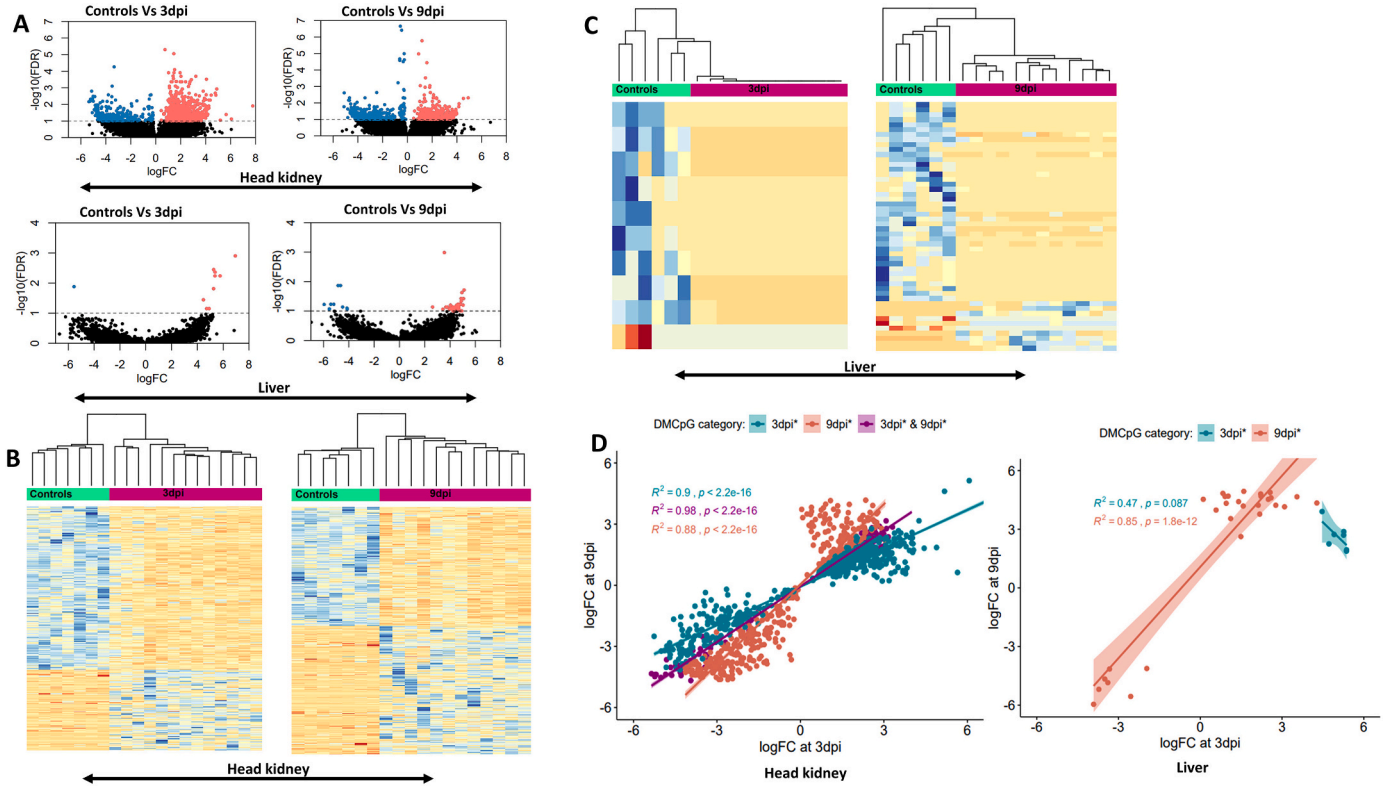

Fig. 3. Differential methylation induced by *P. salmonis* infection; A) Volcano plots showing differential methylation between infected and unchallenged control animals at 3 and 9 days post infection. Significance at False discovery rate < 0.1. B) Heatmaps showing hierarchical clustering samples based on the differentially methylated sites in the head kidney. C) Heatmaps showing hierarchical clustering samples based on the differentially methylated sites in the liver. D) Correlation plots showing correlation between the difference in methylation at 3 days and 9 days post infection in the two tissues where 3dpi* = DMCpGs at 3 dpi, 9dpi* = DMCpGs at 9 dpi and 3dpi* & 9dpi* = DMCpGs at both 3 dpi and 9 dpi.

study by Moraleda et al. [36], where the transcriptomes of both organs showed important changes upon infection, and may be due to the role of the head kidney in immune cell differentiation. Hierarchical clustering (heatmaps) based on the DMCpGs methylation levels showed a consistent methylation trend within samples of each group (Fig. 3B, and C), hence suggesting that the changes in methylation are biologically meaningful and play a role in the response to *P. salmonis* infection. Additionally, methylation differences between 3 dpi and 9 dpi were negligible in both tissues, and the fold changes of the DMCpGs at 3 dpi and 9 dpi relative to control samples generally showed a high positive correlation (Fig. 3D). This observation suggests that methylation patterns change following infection, but less so during the course of the infection. Interestingly, annotation of the differentially methylated sites revealed that some of the DMCpG sites were located within methylation related genes (*DNMT3A, SMYD2* and *METTL21B*) and demethylation genes (*KDM5A* and *KDM2A*, *KDM4B*) (Supplementary file 3). In the head kidney we observed differential methylation in *DNMT3A* which encodes for DNA (cytosine-5)-methyltransferase 3 alpha and is responsible for initiating de novo DNA methylation [1]. *DNMT3A* showed increased methylation in the head kidney tissue of infected fish relative to the healthy animals. Previously significant decreased expression of this gene in the head kidney tissue of *P. salmonis* infected fish relative to healthy fish [36]. Modulated activity of this gene in response to *P. salmonis* infection could be responsible for the changes in methylation patterns observed in the head kidney.

3.5. Functional enrichment of DMCpGs induced by P. salmonis infection

The DMCpGs were assigned to genes according to their position in the annotated genome, and a functional enrichment analysis was performed for each tissue separately (Supplementary file 4). In head kidney, key pathways related to innate and adaptive immunity showed significant enrichment, including regulation of actin cytoskeleton, MAPK signaling pathway, Fc gamma R-mediated phagocytosis and extracellular matrix (ECM) receptor interaction (Fig. 4). In addition, we identified other interesting pathways with substantial numbers of differentially methylated genes including PI3K-Akt signaling pathway, T cell receptor signaling pathway, bacterial invasion of epithelial cells, B cell receptor signaling pathway, phagosome, Toll-like receptor signaling pathway, lysosome, NOD-like receptor signaling pathway and RIG-I-like receptor signaling pathway (Fig. 4). Some of the key differentially methylated genes identified in the current study included *ITGA5, CRKII, PIK3R3, PIK3R1, VAV3, VAV2, JNK, ITGB5* and *GRB2*, which are involved in most of the identified pathways (Fig. 4). Some of these pathways were also identified in the gene expression analyses from the same dataset described by Moraleda et al. [36] and in previous genome-wide association studies (GWAS) for *P. salmonis* resistance in different salmonid species [65,66]. For example, *P. salmonis* has been shown to induce cytoskeletal reorganization of host immune cells via actin depolymerisation [67]. Considering the large number of genes showing differential methylation in the actin cytoskeleton pathway (Fig. 4), it is plausible that the bacteria is actively altering the regulation of this pathway via methylation.

3.6. Differential methylation between resistant and susceptible fish

Comparison of the methylation levels of *P. salmonis* resistant and susceptible fish (based on divergent EBVs) revealed 93 DMCpGs in the head kidney (42 hypermethylated and 51 hypomethylated in resistant fish relative to susceptible) and 81 in the liver (37 hyper-methylated and 44 hypomethylated in resistant fish relative to susceptible) (Fig. 5A, Supplementary file 5). The methylation patterns for these sites were

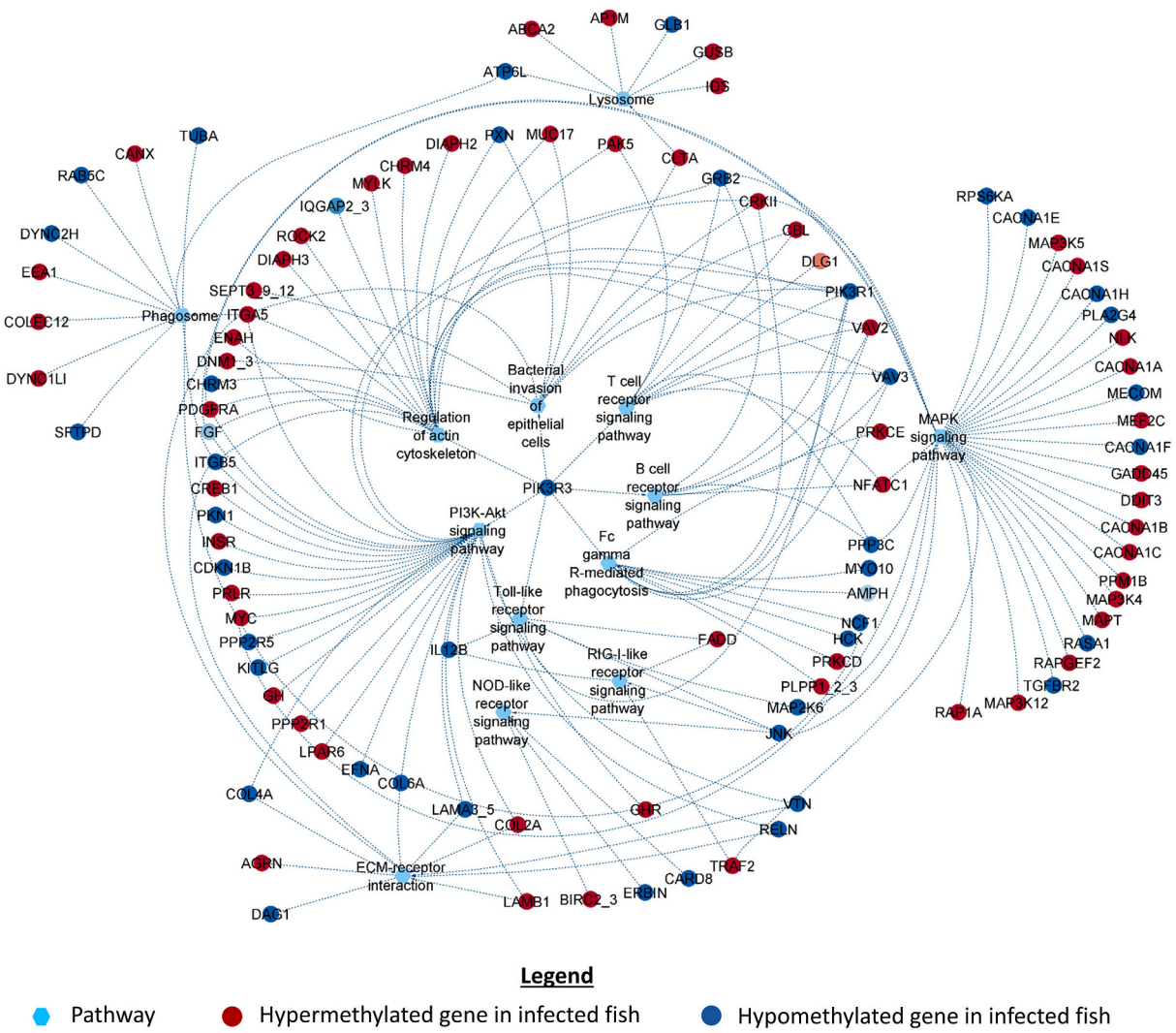

Fig. 4. Interactions between differentially methylated genes and KEGG pathways; The figure shows the relationships between enriched KEGG pathways (light blue) and genes showing increased (red) or decreased (dark blue) methylation in infected fish (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relatively consistent across susceptible and resistant animals (Fig. 5B), suggesting that methylation of these sites might have a genetic basis associated with resistance to SRS. Some of the DMCpGs identified in the head kidney tissue are located within or neighboring well-known immune related genes. Particularly interesting are *TUBA1A, CFL2* and *MTSS1,* which are involved in cytoskeleton structure, and rearrangement regulation [68–70], and may relate to the previously identified enrichment of the actin cytoskeleton pathway. *DNMT3A* was also found differentially methylated between resistant and susceptible samples, showing decreased methylation levels in resistant fish in the in the liver tissue. *DNMT3A* offers a clear link between genetic resistance and differential methylation levels and is a particularly interesting gene for further studies aiming to link differential methylation with genetic resistance to SRS.

4. Discussion

In the current study, the methylation patterns of head kidney and liver in Atlantic salmon and their remodelling in response to *P. salmonis* infection were investigated. Our study demonstrated significant methylation differences between the two tissues. Generally, methylation, especially in the promoter and at the start and the end of transcription regions, is negatively correlated with gene expression, although a significant number of sites showed positive correlation. Infection by *P. salmonis* induced important changes on the head kidney methylome, while methylation in the liver was practically unchanged. The genes showing changes in methylation are involved in several key innate and acquired immune pathways, with the cytoskeleton pathway showing especially consistent methylation changes. Significant differences in methylation between resistant and susceptible fish were also observed, including for the de novo methylation enzyme DNMT3A. Our findings suggest that DNA methylation plays a role in coordinating the Atlantic salmon immune response to *P. salmonis* infection.

The noticeable differences between the methylomes of head kidney and liver are a reflection of the role of methylation in shaping organ and cell differentiation during development [71], which allow for function specialization of the different tissue types and organs. Differing methylation signatures between different tissues or cell types have previously been reported in mammalian [72,73], avian [74] and fish species [9]. In both tissues, CpG sites in intragenic and intergenic regions showed high levels of methylation, whereas in promoter regions methylation showed more variation, with comparable numbers of highly and lowly methylated CpG sites. Similar methylation patterns have been observed in the gill of Atlantic salmon [75] and in the testis, muscle, liver and spleen of European sea bass (*Dicentrarchus labrax*) [9]. The high methylation levels in both intragenic and intergenic regions is

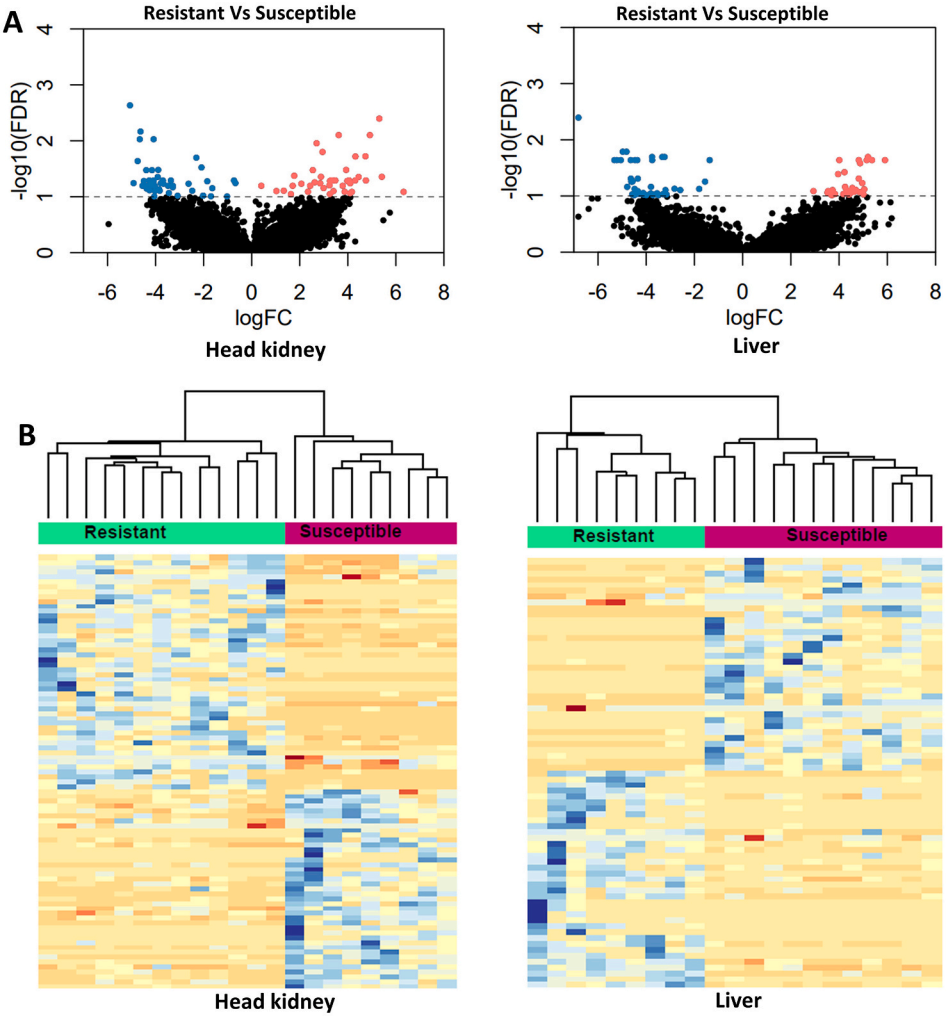

Fig. 5. Differential methylation between genetic resistant and susceptible animals; A) Volcano plots showing differential methylation between resistant and susceptible animals in head kidney and liver, B) Heatmaps showing hierarchical clustering of head kidney and liver samples based on the differentially methylated sites.

thought to contribute to the transcriptional repression of non-coding RNAs and repetitive genomic elements in these regions, hence maintaining genome stability [76]. Additionally, methylation within intragenic regions has been shown to repress the use of alternative transcription start sites that would lead to the expression of transcripts [77] that could be detrimental or non-functional in the cell [78,79]. On the other hand, the bimodal distribution observed in the promoter-TSS regions is consistent with the highly conserved architecture of promoters in vertebrate genomes, which contain two classes of promoters - one with high number of CpGs that are hypomethylated and broadly expressed, and another one with few CpGs that are hypermethylated and generally tissue-specific [80].

The strong negative correlation between gene expression and level of methylation in the 1st exon observed in this study is consistent with previous observations in human cells [10,11] and sea bass testis [9]. These observations deviate from the canonical view where gene expression is regulated through modulation of the DNA methylation of promoter regions [11]. The exact biological mechanisms underpinning the strong negative influence of 1st exon methylation levels remain largely unknown. However, in vertebrates, CG content peaks at the 1st exon / 1st intron junctions [81], and it is now known that the 1st exon is a hotspot for the recruitment of transcriptional factors to initiate transcription. Finally, methylation at the transcription termination sites also showed a noticeably strong significant negative correlation with gene expression, and this could be due to overlap with downstream enhancers [82,83].

In any case, significant correlations between gene expression and methylation levels were observed for sites in all genomic features, indicating that methylation modulation of gene expression is not restricted to the promoter regions in the genome as classically regarded [11]. The correlations were predominantly negative (60%), meaning that in Atlantic salmon increasing methylation will generally result in a reduction of gene expression, in agreement with previous reports in fish and humans [9,84]. However, positive correlations between gene expression and methylation were also observed, implying that for a considerable number of genes increasing methylation would increase expression. These positive correlations between methylation and gene expression have been reported in multiple vertebrate studies [9,84–86], but the mechanism underlying these positive correlations remains poorly understood, althought methylation levels have been observed to promote the binding of some transcription factors [87,88]. Nonetheless, it is clear that the classical model where DNA methylation represses gene expression does not hold for all genes.

Pathogen infections usually trigger host immune responses, which can lead to the activation of different immune cell types through changes in their gene expression [5,6,89]. DNA methylation is one of the well coordinated epigenetic mechanisms that contributes to the transcriptional reprogramming of host immune cells [5,6,89]. Interestingly, through millions of years of coevolution with their hosts, some pathogens have evolved the ability to modulate the expression of host immune

genes via DNA methylation to promote their survival and multiplication in the host cells [89,90]. Our results demonstrate that *P. salmonis* infection reshapes the head kidney methylome in Atlantic salmon, while inducing limited changes in the liver methylation profile. These results contrast with the previous transcriptomic study on the same population, where Moraleda et al. reported substantial changes in both the head kidney and liver transcriptome in response to *P. salmonis* [36], and therefore other epigenetic mechanisms must be modulating the liver transcriptional response to the bacteria. This difference is probably associated with the role of the head kidney, a primary lymphoid organ where fish immune cells go through differentiation and maturation [91,92]. Interestingly, we observed negligible methylation differences between infected animals at the two infection time points and this implies that the methylation landscapes established at the onset of the infection are maintained through the course of the infection.

Methylation in the head kidney targeted several specific pathways. The most interesting is possibly the actin cytoskeleton pathway, which modulates a wide range of processes within the cell, including phagocytosis, endocytosis, intercellular interaction, cell division, intercellular signal transduction, cell movement and morphology [93]. The cytoskeleton plays an important role during SRS infection. Previously, Ramirez et al. demonstrated that *P. salmonis* infection of Atlantic salmon macrophages disrupts cytoskeleton disorganization and increases the cells' actin synthesis, which the bacteria utilize to generate vacuoles where they survive and multiply while shielding from cytosolic detection and destruction [67]. Upon *P. salmonis* infection, we detected differential methylation of 20 genes involved in the actin cytoskeleton regulation. Interestingly, two of these genes (*ITGA5* and *CRKII)* showed strong negative correlation (−0.62 and − 0.64, respectively) between their expression and methylation level, and had been previously identified as differentially expressed in head kidney between infected and non-infected Atlantic salmon fish [36]. *ITGA5* encodes for the alpha and beta subunits of integrin, which is a transmembrane receptor that modulates intracellular cytoskeleton organization [94]. *CRKII* encodes an adapter molecule crk that plays crucial roles in actin reorganization, phagocytosis, lymphocyte adhesion, activation and migration [95–97]. Previous GWAS for *P. salmonis* resistance in different salmonid species, including Atlantic salmon, coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*), have also highlighted actin cytoskeleton as an important mechanism of the host response to infection [65,66]. Altogether, these results demonstrate the importance of both genetic variants and DNA methylation on the modulation of actin cytoskeleton in response to *P. salmonis* infection.

We observed differential methylation of immune related genes between *P. salmonis* resistant and susceptible fish in the liver and head kidney. Interestingly some of these genes were also connected to cytoskeleton regulation (i.e. *TUBA1A, CFL2* and *MTSS1* in the head kidney tissue, and *DOCK1* in the liver). These genes and the others previously identified showing differential methylation between control and infected samples clearly point to the regulation of the cytoskeleton pathway via methylation being an important mechanism during *P. salmonis* infection. Additionally, in the liver *DNMT3A*, a gene that encodes for a major de novo DNA methylation enzyme [1], was differentially methylated between susceptible and susceptible fish. Together, these results of differentially methylated genes upon infection and between fish with divergent susceptibility could potentially be used to improve genomic selection for Atlantic salmon resistance to *P. salmonis* infection. In addition, some of the differentially methylated genes in our study play critical roles in immune response, and they may allow the design of new gene specific therapeutic drugs against *P. salmonis* infection in Atlantic salmon.

A large number of differentially methylated sites did not show significant association with gene expression and did not overlap with differential gene expression, a phenomenon also observed in a previous similar study in coho salmon (*Oncorhynchus kisutch*) [49]. These observations may be attributed to the bulk nature of DNA methylation and transcriptome profiling, where cell diversity is not considered. The diverse cell populations in these tissues probably undergo unique DNA methylation and transcription alterations upon *P. salmonis* infection, lost via bulk tissue sequencing. Consequently, this diversity can only be investigated via single cell transcriptome and DNA methylation sequencing. In addition, as gene expression is a multifactorial trait, where multiple regulatory mechanisms including histone modification, long and small non-coding RNAs, etc. play critical roles in modulating transcription levels, it would be interesting to assess their involvement in the Atlantic salmon response to *P. salmonis* and their cross-talk with DNA methylation. It is also worth highlighting that the control animals used in the current study did not receive a sham injection at the time when the rest of the animals were inoculated, and therefore the response to injection cannot be discriminated from the response to the bacteria. However, our results show methylation differences in immune genes that have been previously highlighted as associated to *P. salmonis* infection, indicating that indeed the revealed differences between infected and healthy animals are mainly due to the response to the pathogen.

5. Conclusions

In the current study, we investigated the DNA methylation patterns in the liver and head kidney of Atlantic salmon, as well as the methylation changes triggered by *P. salmonis* infection. Head kidney and liver present organ-specific methylation patterns, however the distribution of methylated sites across gene features and the methylation gene expression trends are similar in both tissues. Although methylation was mostly negatively correlated with gene expression, there were a moderate number of positive correlations. Nonetheless, overall methylation towards the start and the end of the gene was associated with reduced expression. *P. salmonis* infection induced significant changes in the methylome of the head kidney, while the liver remained almost unaltered. These methylation changes regulated genes involved in crucial immune response pathways such as actin cytoskeleton regulation, pathogen recognition and phagocytosis. Comparison between resistant and susceptible fish highlight methylation of the actin cytoskeleton as a potential mechanism involved in genetic resistance to SRS. Further, the de novo methylation gene *DNMT3A* showed significant differences in methylation, suggesting a direct explanation for the observed differences in control vs infected and resistant vs susceptible, offering an excellent target for further studies. These results contribute to the growing knowledge on the immune response of Atlantic salmon to *P. salmonis* infection and may provide future avenues for the development of more targeted therapeutic strategies.

Ethics declarations

The challenge experiment was carried out following the local and national regulatory systems and was approved by the Animal Bioethics Committee (ABC) of the Faculty of Veterinary and Animal Sciences of the University of Chile (Santiago, Chile), Certificate No. 01–2016, that based its approval decision on the Council for International Organizations of Medical Sciences (CIOMS)standards, in accordance with the Chilean standard NCh-324-2011.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Data availability

All sequence data generated for this study have been submitted to the NCBI's BioProject database under accession number PRJNA522369.

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Authors' contributions

Conceptualization: DR, RDH, JMY; Formal analysis: RM, CP, AG, DR; Project administration: RDH, JMY; Writing - original draft: RM, DR, RDH; Writing - review & editing: All authors.

Declaration of Competing Interest

Authors declare no competing interests.

Data availability

All data is available in public repositories or within the manuscript

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2022.110503.

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