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Identification of novel biomarkers of inflammation in Atlantic salmon (*Salmo salar* L.) by a plasma proteomic approach

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ABSTRACT

Monitoring fish welfare has become a central issue for the fast-growing aquaculture industry, and finding proper biomarkers of stress, inflammation and infection is necessary for surveillance and documentation of fish health. In this study, a proteomic approach using mass spectrometry was applied to identify indicators of the acute response in Atlantic salmon blood plasma by comparing *Aeromonas salmonicida* subsp. *salmonicida* infected fish and non-infected controls. The antimicrobial proteins cathelicidin (CATH), L-plastin (Plastin-2, LCP1) and soluble toll-like receptor 5 (sTLR5) were uniquely or mainly identified in the plasma of infected fish. In addition, five immune-related proteins showed significantly increased expression in plasma of infected fish: haptoglobin, high affinity immunoglobulin Fc gamma receptor I (FcγR1, CD64), leucine-rich alpha 2 glycoprotein (LRG1), complement C4 (C4) and phospholipase A2 inhibitor 31 kDa subunit-like protein. However, various fibrinogen components, CD209 and CD44 antigen-like molecules decreased in infected fish. Selected biomarkers were further verified by Western blot analysis of plasma and real time PCR of spleen and liver, including CATH1, CATH2 and L-plastin. A significant increase of L-plastin occurred as early as 24 h after infection, and a CATH2 increase was observed from 72 h in plasma of infected fish. Real time PCR of selected genes confirmed increased transcription of *CATH1* and *CATH2*. In addition, serum amyloid A mRNA significantly increased in liver and spleen after bacterial infection. However, transcription of L-plastin was not consistently induced in liver and spleen. The results of the present study reveal novel and promising biomarkers of the acute phase response and inflammation in Atlantic salmon.

1. Introduction

Aquaculture has contributed to the impressive growth of seafood supply for human consumption. The surveillance of the health status and welfare of farmed fish is important for sustainable production. To date, the concepts and definitions of fish welfare are still in development, as well as the relevant objective measuring methods (Raposo de Magalhães et al., 2020).

Inflammation is a protective response of the immune system to harmful stimuli, including infection, tissue injury, tissue stress and malfunction, and other non-microbial exogenous inducers (Medzhitov, 2008). Recent research in mammals indicate that stress also can activate the inflammatory response in the brain, namely central inflammation or neuroinflammation, as well peripheral inflammation (Rohleder, 2014). Increasing evidence confirm that the inflammatory response is universally found as 'common soil' in multifactorial diseases, including cardiovascular and metabolic diseases, psychotic neurodegenerative disorders and cancer (Scrivo et al., 2011). For fish, inflammatory response could also be induced by improper diet components (Kortner et al., 2016), vaccine adjuvants (Noia et al., 2014) and various xenobiotics in water, such as microplastics, heavy metals, algae toxin and other environmental chemicals (Jing et al., 2020; Lin et al., 2020; Lu et al., 2016).

The acute phase response (APR) is an early innate immune response initiated by inflammatory signals, leading to the release of acute phase proteins (APPs) to the bloodstream to re-establish homeostasis following infection or tissue damage. Although the APPs are important early diagnostic indicators of disease in human and veterinary medicine (Eckersall and Bell, 2010), these APPs are less studied in fish and the majority of studies describe the APR at gene level. In addition, the real

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functions of these APP homologues in fish are not always known or consistent with the function of their mammalian counterparts. For instance, serum amyloid A (SAA) is an important APP in both pets and farm animals and has been recognized as an APP in fish for a long time (Jensen et al., 1997). However, SAA protein in fish plasma is difficult to detect and its existence in plasma still remains unclear. Furthermore, the mRNA of other well-established mammalian APP, including five C-reactive protein (CRP)/serum amyloid P component (SAP) like molecules, remained unaltered in Atlantic salmon after *A. salmonicida* infection (Lee et al., 2017). Thus, the functional established APPs in mammals may play different roles in fish, and presently unknown fish APPs may have a more central role.

Plasma contains an extraordinary diverse set of proteins, which differ in over 10 orders of magnitude in abundance (Anderson and Anderson, 2002). Liquid chromatography tandem mass spectrometry (LC-MS/MS) is one of the mostly used tools to identify plasma proteins, due to its high versatility and protein identification potential in complex samples. Advances in LC separation power and MS resolution scan rate enable the profiling of more than 15,000 proteins (>12,000 genes) from mammalian tissue samples (Mertins et al., 2016). MS has been applied in profiling plasma proteins in salmonid fishes, including the documentation of the plasma proteome response during sea water adaptation (Morro et al., 2020), following immunization with protein as antigen (Bakke et al., 2020), following infection with salmonid alphavirus (Braceland et al., 2013) and following anti-sea lice treatment with chemical azamethiphos in rainbow trout (Barisic et al., 2019). MS was also used in analyzing proteomic response in plasma of other fish species, such as identifying chronic stress (overcrowding, handling and hypoxia) in treated gilthead seabream (Raposo de Magalhães et al., 2020), and the response in bacteria infected tilapia (Charlie-Silva et al., 2019) and virus infected zebrafish (Medina-Gali et al., 2019). The proteomic profiling after bacterial infection has been investigated in kidney and liver of salmon (Causey et al., 2018; Liu et al., 2017), however to the authors' knowledge there is no report on plasma proteomic response in salmonid fish after bacterial infection.

The aim of this study was to characterize changes in the plasma proteome of Atlantic salmon following an acute bacterial infection using an unbiased shotgun proteomic approach. We describe the discovery of both novel and promising biomarkers for verification of inflammation in Atlantic salmon. The identified candidates would be useful in further developing convenient and non-lethal diagnostic tools to access the health and welfare condition of farmed fish.

2. Materials and methods

2.1. Experimental setup and sampling

Experimental fish originated from brood stock (SalmoBreed standard strain) screened and verified negative for ISAV, SPDV, PRV and IPNV by qPCR at a qualified laboratory. The experimental trial was carried out at VESO Vikan research facility (Namsos, Norway). VESO Vikan is operated in compliance with the OECD principles of Good Laboratory Practice (GLP), EU Council Directive 2004/10/EC and Guidelines to Good Manufacturing Practice issued by the European Commission, cf. Directives 2003/94/EC and 91/412/EC. Acclimatization was carried out according to standard operating procedures at VESO Vikan. A total of 170 Atlantic salmon smolts at an average weight of 100 g were kept in seawater tanks with a volume of 500 L, a flowthrough system and a maximum stocking density of 40 kg/m3. Seawater was collected from 60 m depth, filtrated and UV-treated before added to the tanks. The effect of the UV treatment on water quality is regularly monitored at the facility. Fish were kept at a temperature of 12 ± 1 °C and with a photoperiod regime of L:D = 12:12. The fish and tanks were tended and monitored daily by staff at VESO Vikan. Fish were starved for 24 h prior to intraperitoneal (IP) injection with 10^4 colony forming units (CFU) (0.1 ml per fish) of *Aeromonas (A.) salmonicida subsp. salmonicida* per individual (infected fish, n = 90). Control, non-infected fish were kept in a separate tank (n = 70) (hereafter controls). Fish were anesthetized (MS222, Benzoak) and euthanized with a blow to the head just prior to sampling. Samples including blood, spleen and liver were collected at 0 h (h) (n = 10), which is equal to just prior to challenge, and at 12h, 24h, 48h, 72h, 96h, and 7 days after challenge (n = 15 and n = 10 for infected fish and controls at all time points, respectively). Blood samples were collected from the caudal vein in tubes with EDTA and immediately centrifugated, and plasma collected in separate tubes and stored at −20 °C until use. Organ samples of liver and spleen were collected in separate tubes with RNA*later*, stored at +4 °C overnight followed by storage at −20 °C until use.

2.2. Sample preparation and mass spectrometry analysis

Selected plasma samples were analyzed by mass spectrometry (20 infected samples, 8 control samples) in two separate rounds. Protein concentrations of samples were determined by fluorometry (Qubit Fluorometer 2.0) and volume corresponding to 30 μg protein was diluted to 25 μL in ammonium bicarbonate buffer (100 mM), and denatured by adding equal volume of 2,2,2-trifluoroethanol (Sigma), and 1,4-dithiotreitol (DTT, 2.5 μl, 200 mM, Sigma) before incubation with shaking at 90 °C for 20 min. To alkylate cysteine residues, iodoacetamide (IAM, 10 μl, 200 mM, Sigma) was added and samples were incubated in the dark at room temperature for 1 h. DTT (2.5 μL) was added to inactivate surplus IAM, followed by incubation in the dark at room temperature for 1 h. Ammonium bicarbonate (400 μl, 25 mM, Fluka) was added before samples were digested by trypsin (1:35 enzyme:protein weight-ratio, Sequence grade modified trypsin, Promega, Madison, WI) and desalted by Pierce C18 spin columns (Thermo Scientific), following instructions from manufacturer. Samples representing 0.5 μg protein as tryptic peptides dissolved in 2% acetonitrile (ACN, Merck, Germany) with 0.5% formic acid (Merck) were injected into an Ultimate 3000 RSLC system (Thermo Scientific) connected online to a Q-Exactive HF mass spectrometer (Thermo Scientific) equipped with EASY-spray nano-electrospray ion source (Thermo Scientific) for a 120 min ACN gradient (5–90% ACN). Samples were loaded and desalted on a pre-column (Acclaim PepMap 100, 2 cm × 75 μm ID nanoViper column, packed with 3 μm C18 beads, Thermo Scientific) at a flow rate of 5 μl/min for 5 min with 0.1% trifluoroacetic acid (Merck).

Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps at a flow rate of 250 nl/min on a 25 cm analytical column (PepMap RSLC, 25 cm × 75 μm ID EASY-spray column, packed with2 μm C18 beads with pore size 100 Å). Solvent A and B were 0.1% FA (vol/vol) in water and 100% ACN, respectively. The gradient composition was 5% B during trapping (5min) followed by 5–7% B over 0.5min, 7–22% B for the next 59.5min, 22–35% B over 22 min, and 35–90% B over 5min. Elution of very hydrophobic peptides and conditioning of the column were performed during 10 min isocratic elution with 80% B and 15 min isocratic elution with 5% B.

The eluting peptides from the LC-column were ionized in the electrospray and analyzed by the Q-Exactive HF. The mass spectrometer was operated in the DDA-mode (data dependent acquisition) to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Q Exactive HF Tune 2.9 and Xcalibur 4.1 Survey full scan MS spectra (from m/z 375–1500) acquired in the Orbitrap with resolution R = 120 000 at m/z 200, automatic gain control (AGC) target of 3e6 and a maximum injection time (IT) of 100 ms. The 12 most intense eluting peptides above an intensity threshold of 50 000 counts, and charge states 2 to 5, were sequentially isolated to a target value (AGC) of 1e5 and a maximum IT of 110 ms in the C-trap, and isolation width maintained at 1.6 m/z (offset of 0.3 m/z), before fragmentation in the HCD (Higher-Energy Collision Dissociation) cell. The minimum AGC target for fragmentation were set at 5.5e3. Fragmentation was performed with a normalized collision energy (NCE) of 28%, and fragments were detected in the Orbitrap at a resolution of 60 000 at m/z 200, with

first mass fixed at m/z 120. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 20s with "exclude isotopes" on. Lock-mass internal calibration (m/z 445.12003) was enabled. The spray and ion-source parameters were as follows: Ion spray voltage = 1800 V, no sheath and auxiliary gas flow, and capillary temperature = 275 °C.

2.3. Processing of proteomic data

In total, 28 samples were submitted to proteomic analysis, at two separate time points. Each sample was analyzed by MS a single time and in random order, with one sample included in both MS runs (IP 72h ID1). The samples were divided in three groups: control samples (n = 8); infected fish at time 0h (n = 3); and samples from infected fish at later time points (n = 17).

The raw data was converted to Mascot Generic Format (mgf) peak lists with MS convert with peak picking of MS2 to convert to centroid data (Chambers et al., 2012). Peak lists obtained from MS/MS spectra were identified using X!Tandem (X!Tandem Vengeance, v2015.12.15.2). The search was conducted using SearchGUI (v3.3.13). Protein identification was conducted against a concatenated target/decoy database of *Salmo salar* proteome UP000087266 (reference proteome downloaded from UniProtKB in April 2019; 82380 sequences). Interference from *A. salmonicida* (476 reviewed proteins, downloaded from UniProtKB in April 2019) (Apweiler et al., 2004) and common contaminants (cRAP database, version 2012.01.01) were included to rule out confounding protein identities. Decoy sequences were created by reversing the target sequences in SearchGUI (Barsnes and Vaudel, 2018). The identification settings were as follows: specific trypsin digest with a maximum of 2 missed cleavages; 10 ppm as MS1 and 0.02 Da as MS2 tolerances; fixed modifications: Carbamidomethylation of C (+57.021464 Da); variable modifications: Oxidation of M (+15.994915 Da); fixed modifications during refinement procedure: Carbamidomethylation of C (+57.021464 Da). Peptides and proteins were inferred from the spectrum identification results using PeptideShaker (v1.16.38) (Vaudel et al., 2015). Peptide spectrum matches (PSMs), peptides and proteins were validated at a 1.0% false discovery rate (FDR) estimated using the decoy hit distribution. All samples were processed in parallel in PeptideShaker to provide data for all identified proteins across all samples, with individual quantitative measures for each sample. The number of validated peptide-spectra-matches allocated to a protein in a given sample, was used for label-free quantitative evaluation.

Quantified proteins were sample corrected by division of the summed sample spectra followed by variance stabilized normalization, and differential expressed proteins (DEPs) were identified using reproducibility-optimized test statistic (ROTS) (Huber et al., 2002; Suomi et al., 2017), where infected samples (12h, 24h, 48h, 72h) were compared with control samples (0h, 12h, 24h, 72h) plus samples from infected group collected at time 0h. Significant proteins were defined as having a p-value below 0.01. Undetected proteins were treated as NaN. The resulting DEPs were visualized using probabilistic PCA (Porta et al., 2005), and by heatmap using the Seaborn Python library (version 0.10.0).

Proteins only identified in infected fish at 24h, 48h, 72h or 7 days was identified by comparing the accession numbers of all proteins with reported PSMs for this group compared to control samples and fish from the infected group at time 0h.

2.4. Quantitative real-time PCR (qPCR) analysis of gene expression

To further study the kinetics of potential indicators found in proteomic analysis, CATH1, CATH2 and L-plastin were selected for qPCR since they are molecules directly involved in immune response. Although SAA was not detected in proteomic analysis, it was included in qPCR since it has been recognized as acute response indicator at transcriptional level. RT-qPCR was performed on liver and spleen samples (n = 5) from each group (0h, infected and controls) and each time point. A sample size of 10 μg was excised and transferred to a tube with 2 M DTT (Bio-Rad) in RLT buffer (Qiagen) and a 5 mm TissueLyser bead (Qiagen). Samples were lysed at 25 Hz for 5 min, centrifuged at 21 000 g for 3 min at 4 °C, and the homogenate was transferred to safe-lock tubes and stored at −80 °C. Homogenate was thawed in 37 °C water bath, and RNA was isolated automatically using a QIAcube and RNeasy mini QIAcube kit (Qiagen) according to manufacturer's protocol, using 70% EtOH for spleen samples and 50% EtOH for liver samples and an elution volume of 50 μL. Concentrations were measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher) and RNA stored at −80 °C. cDNA was prepared from RNA using QuantiTect reverse transcription kit (Qiagen), according to manufacturer's protocol. A small volume was removed from a number of samples to prepare a cDNA pool used with primer optimization and efficiency tests. qPCR was performed using Maxima SYBR green qPCR kit (Thermo Fisher), with ROX added to SYBR green master mix to a concentration of 20 nM. Each reaction consisted of 10 μL 2X master mix with 20 nM ROX, 2 μL forward and reverse primer (concentrations optimized), 3 μL DEPC-treated water, and 3 μL cDNA template, for a total reaction volume of 20 μL. All primers were optimized by checking performance at 50, 300, 450, and 900 nM using duplicates of 3 μL 5 ng/μL cDNA pool as template. Efficiency tests were performed on a 5-fold dilution series of cDNA pool, ranging from 50 ng/μL to 3.2 pg/μL, using 2 μL template in triplicates. Conditions of qPCR cycle were as follows: 50 °C/2 min, 95 °C/10 min, followed by 40 cycles of 95 °C/15 s, 60 °C/1 min). Relative quantification of target genes were calculated by comparing expression with reference gene Ef1α and expression before challenge, according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers sets for qPCR are listed in Table 1. Gene transcripts were compared using an unpaired Student's t-test and considered as statistically significant at $p \leq 0.05$.

2.5. Production and purification of His-tagged salmon cathelicidins (CATH1 and CATH2) prepropeptides and L-plastin actin-bundling domain

The cDNA encoding salmon CATH1, CATH2 and one actin-bundling domain of L-plastin were amplified using the primer sets listed in Table 1. Purified PCR fragments were digested with *Eco*RI and *Bam*HI or *Sal*I and *Bam*HI (Thermo Fisher) and ligated into the PQE80L vector (Qiagen). The recombinant plasmids were transformed into *E. coli* TOP10 competent cells (Thermo Fisher). After confirmation by PCR, the purified plasmids of positive clones were subsequently transformed into *E. coli* BL21 (DE3) competent cells (Thermo Fisher). Bacteria containing recombinant plasmid were cultured in Luria–Bertani broth with 50 μg/L kanamycin and induced with 0.4 mM isopropyl β-*d*-thiogalactoside (IPTG) at 37 °C for 12 h. Subsequently, bacteria were harvested by centrifugation at 3000*g* for 30 min, frozen and defrosted, resuspended in B-PER™ bacterial protein extraction reagent (Thermo Fisher), and mixed in a 1:9 vol ratio with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, pH 8.0). The recombinant proteins were purified using the HisPur™ Ni-NTA Superflow agarose (Thermo Fisher), with a washing buffer containing 40 mM imidazole for CATH1, and 30 mM imidazole for CATH2, respectively. The purity of the recombinant proteins was checked on a 4–12% Criterion™ XT Bis-Tris Protein Gel (Bio-Rad), and stained with SimpleBlue SafeStain (Invitrogen). The purified proteins were dialyzed with Slide-A-Lyzer™ G2 dialysis cassettes 3.5K MWCO (Thermo Fisher) in PBS overnight. Protein concentration was measured with a QuickStart Bradford protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

2.6. Production of rabbit anti-salmon polyclonal antibodies

Purified recombinant CATH1 or CATH2 prepropeptide (0.5 mg/ml) was emulsified 1:1 in Freund's complete adjuvant, and 1 ml was injected into a New Zealand white rabbit in Department of Production Animal Clinical Sciences, NMBU. Immunization of rabbits was boosted after 2, 3

Table 1
Primers for real time PCR and gene cloning.

Gene	Accession no.	Forward primer (5′-3′)	Reverse primer (5′-3′)	Usage
EF1α	BG933853	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	qPCR
CATH 1	GQ870278	TGGACCAGTCTGAGGTGTCT	TTGCTAGCCTCCAGAATCGG	qPCR
		GTGAATTCGCAGGACCAAACTGGGACCAGG	AACCTAGGCTATGCAAAGCGATTTCCATC	cloning
CATH 2	XM_014140493	GAGCAGGCTTTCCATCCACT	GGGGGAAGCTCAACCTTACA	qPCR
		GTGAATTCGCAGACCCAGACTGAGACTAGG	AACCTAGGCTATGCATTGCGAGTTCCACC	cloning
LCP1	XM_014165132	GGAGGTCAAACCTAAGATGGTG	TCTTTTCCCTGATTCTGGTTGT	qPCR
		CGCGGATCCGGAACTTCAGAGCAGTCCAGC	CCCGTCGACCTACTTCAGGGCAGGGTACTTGTT	cloning
SAA	NP_001140037	GGTGCTAAAGACATGTGGCG	CCACTGGAACCCTGAACCAT	qPCR

and 4 weeks with the same amount of antigen in Freund's incomplete adjuvant. The final bleeding was performed 6 weeks after the initial immunization and the antiserum was separated from the blood after clotting overnight at 4 °C followed by centrifugation at 3000*g*. The antiserum was aliquoted and stored at −20 °C.

A commercial anti-human L-plastin antibody (Thermo Fisher, Cat. No. PA5-97918) was used to detect salmon L-plastin in blood plasma based on the high similarity of actin-binding domains between human and teleost fish. The recombinant soluble 1st actin-binding domain of salmon L-plastin was used in WB as control.

2.7. Western blot analysis

Salmon serum from three individuals of each group at different time points was diluted 40 times, boiled for 2 min and kept on ice before electrophoresis. Ten μl diluted serum per well were run on 4–12% Criterion™ XT Bis-Tris Protein Gel (Bio-Rad) along with the Precision Plus Protein™ WesternC™ Standard (Bio-Rad). After electrophoresis, Trans-Blot Turbo System was used for protein transfer (Bio-rad). Anti-salmon CATH1 or CATH2 serum at a 1:1000 dilution or anti-L-plastin antibody at a 1:2000 dilution was used as primary antibody followed by horseradish peroxidase-conjugated donkey anti-rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:5000 as secondary antibody. Pierce ECL western blotting substrate (Thermo Fisher) was used for enhanced chemiluminescence.

3. Results

3.1. Proteomics

In total, 28 samples were submitted to proteomic analysis, and 657 proteins present with more than 1 peptide were identified from 14,258 peptides matched to 414,797 spectra. Contaminants included 12 identified proteins from the cRAP list, and these were present in low amounts (561 of 414,797 of annotated spectra across all samples). No protein from *A. salmonicida* was identified. The admixture of non-salmon proteins was deemed negligible and further data processing was done on a data set processed and based only on the *Salmo salar* Uniprot proteome. The complete list of proteins is provided in supplementary file 1.

Across all samples and conditions, 236 (37%) proteins were identified in all 29 LC-MS/MS runs. Differential expressed proteins (DEP) in infected fish compared with controls and 0-h samples were identified using ROTS (Suomi et al., 2017), with $p < 0.01$ deemed as significant. This yielded a subset of 23 proteins (Table 2 and Fig. 1). Five immune-related proteins showed significantly increased expression in plasma of infected fish: haptoglobin-like molecule, high affinity immunoglobulin Fc gamma receptor I -like molecule (FcγR1, CD64), leucine-rich alpha 2 glycoprotein (LRG1), complement C4 (C4) and phospholipase A2 inhibitor 31 kDa subunit-like protein. However, various fibrinogen components (including different alpha, beta, gamma chains and one fibrinogen-like protein), and CD209 antigen-like and CD44 antigen-like molecules decreased in infected fish.

From 645 proteins, 514 proteins were identified in all three groups (controls, 0h-samples and infected fish), whereas 61 proteins were uniquely or mainly identified in infected fish (at 24h, 48h, 72h and 7 days). (Supplementary file 2). These included Cathelicidin (Q2NNC4_SALSA), L-Plastin (A0A1S3NZ03) and sTLR5 (Q5 UT54) (Fig. 2).

Principal component (PCA) analyses were performed for the identified proteins with differential relative abundance across groups to check how well the samples clustered. Transforming the 23 differentially expressed proteins with a PCA showed that this subset of genes could be used to separate samples from infected fish from the control and 0h samples (Fig. 3). The variance between control and 0h samples was negligible compared with the variance in the IP samples.

Table 2
List of differentially expressed proteins (DEP) when comparing infected fish at 24h, 48h, 72h or 7 days with control, non-infected fish or fish at day of infection (0h) ($p < 0.01$). Accession number in *Salmo salar* database with given protein names, Log2 values for fold change, P-value and FDR (%) is given.

Accession numbers	Fold change (log2)	P-value	FDR	Description
A0A1S3SLR1	−1.58	0.0015	0.0000	CD209 antigen-like protein C isoform X3
A0A1S3SVF8	−1.41	0.0018	0.0833	Fibrinogen-like protein 1
B5X5M2	1.00	0.0092	0.1471	Fatty acid-binding protein, heart
A0A1S3NRI3	1.35	0.0009	0.0000	High affinity immunoglobulin gamma Fc receptor-like isoform X1 (CD64)
C0H9M4	1.01	0.0075	0.1429	Glucose-6-phosphate isomerase
A0A1S3QW80	1.07	0.0072	0.1429	Mannose-specific lectin like
A0A1S3MUZ7	1.01	0.0088	0.1429	Phospholipase A2 inhibitor 31 kDa subunit like
B5X5I8	1.20	0.0098	0.1471	Profilin
A0A1S3MTC5	0.81	0.0046	0.1250	Uncharacterized protein LOC106574835
A0A1S3PTD3	1.12	0.0007	0.0000	Haptoglobin-like
A0A1S3NVC7	1.11	0.0009	0.0000	Haptoglobin-like
A0A1S3KPF0	−0.96	0.0070	0.1429	CD44 antigen-like
A0A1S3N9Y0	−0.78	0.0017	0.0833	Fibrinogen alpha chain-like
A0A1S3NAJ7	−1.23	0.0013	0.0000	Fibrinogen beta chain-like
A0A1S3T3K1	−1.20	0.0014	0.0000	Fibrinogen beta chain-like isoform X2
A0A1S3RF10	−1.06	0.0010	0.0000	Fibrinogen gamma chain-like
A0A1S3SJC9	−1.13	0.0012	0.0000	Fibrinogen gamma chain-like
A0A1S3PA95	−0.34	0.0045	0.1250	Proteoglycan 4-like isoform X1
A0A1S3NJZ7	−0.92	0.0063	0.1250	A disintegrin and metalloproteinase with thrombospondin motifs 13
A0A1S3RIB5	−0.34	0.0084	0.1429	Coagulation factor IX
A0A1S3M844	0.57	0.0018	0.0833	Leucine-rich alpha-2-glycoprotein-like
A0A1S3QML7	0.46	0.0014	0.0000	Complement C4-like
A0A1S3RYT8	0.37	0.0046	0.1250	Complement C4-like

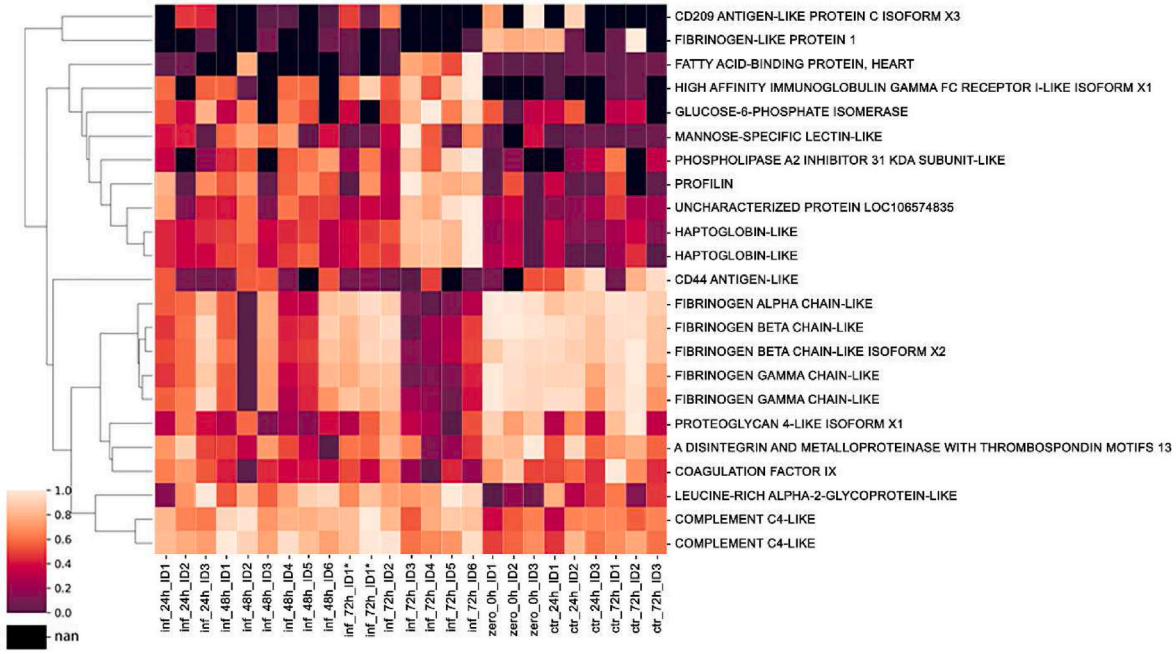

Fig. 1. Heat map of scaled data, for proteins where DEP $p < 0.01$. Rows represent expression patterns of individual proteins, while each column corresponds to a biological replicate (fish). * = same samples analyzed at two time points. Spectral data for each protein was normalized for each sample, and proteins were transformed to a scale from 0 to 1, where 1.0 is the highest abundance (white) and 0.0 is the lowest abundance (dark purple). Missing values are shown by black (nan – Not a number).

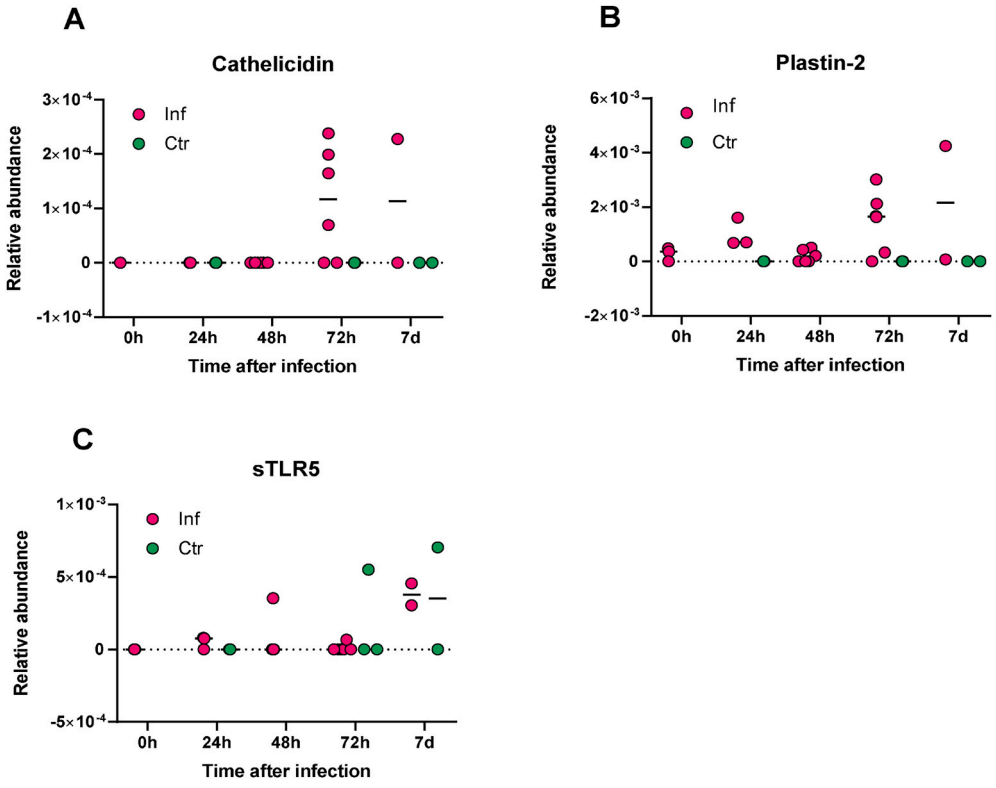

Fig. 2. Proteins uniquely expressed in infected fish. Relative abundance of identified proteomic spectra for Cathelicidin (**A**), Plastin-2 (**B**) and sTLR5 (**C**) in relation to total number of identified spectra in individual samples. Circles represent individual samples (infected and controls) and the dotted line represent the mean value of all samples.

3.2. RT-qPCR of selected candidate genes

Transcription of *CATH1* and *CATH2* increased significantly in the liver from 24 h after infection, and consistently increased transcripts of *CATH1* (>500 times) and *CATH2* were detected from 72 h to end of trial (Fig. 4). In spleen, significant increased *CATH1* and *CATH2* mRNA

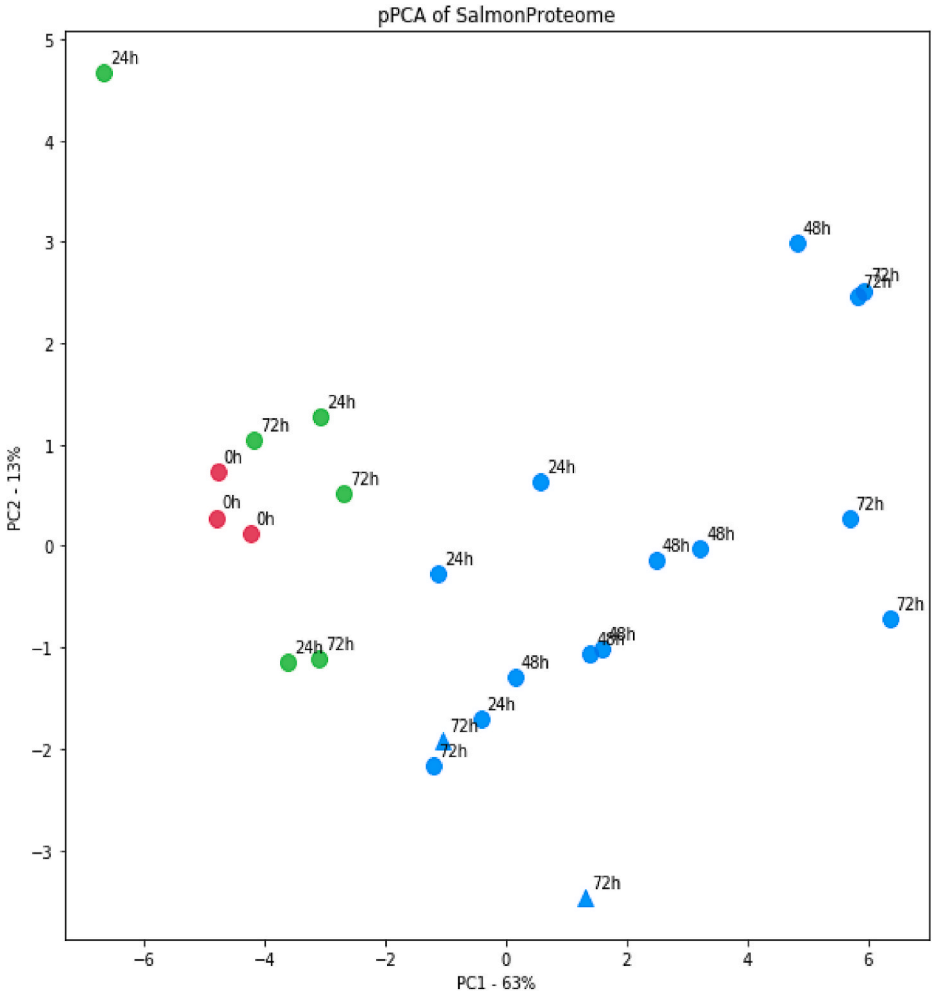

Fig. 3. PCA plot based on the 23 differentially expressed proteins ($p < 0.01$) for the 29 individual sample runs (one sample analyzed in duplicate in both analytical runs is shown as triangles). Each point is labelled with time for sampling for control fish (green), 0h-samples (red) and infected fish at later time points (blue).

transcription started at 24 h post-infection, with peak transcription of *CATH1* at 48 h (110 times) and *CATH2* at 7 d after bacterial infection.

L-plastin *(LCP1)* transcription only showed a minor fold increase in both organs after bacterial infection (Fig. 4). In liver, the transcription of *SAA* increased significantly at 12 h post-infection, and a dramatically increased transcription (>1000 times) could be observed from 48 h to 7 d after bacterial infection (Fig. 4). In spleen, Increased *SAA* mRNA was observed from 24 h (20 times).

3.3. Western blot analysis

To validate the results of the proteomic analysis, the presence of CATH1 and CATH2 (Fig. 5) and L-plastin (Fig. 6) proteins in plasma were further analyzed by Western blot, using polyclonal rabbit anti-salmon cathelicidin antibodies and a commercial anti-human L-plastin Ab, respectively. Considering the antimicrobial potential and the small molecular size of the mature peptides, the full CATH1 and CATH2 pre-propeptides were used in the recombinant protein production. Both salmon CATH prepropeptides were produced as soluble protein in *E. coli* (Fig. 5A). The mature peptide of CATH2 could be detected from 72 h in infected fish (Fig. 5B). An increased expression of mature peptide of CATH1 started from 72 h, but a weak expression could also be found in some individuals in the control group.

The anti-human L-plastin Ab could recognize the recombinant salmon L-plastin actin-bundling domain as shown in Fig. 6. A protein with MW 70 kDa which matches the monomeric salmon L-plastin could be detected in blood plasma of individuals in both groups, whereas increased signal of multiple proteins or protein complex (MW larger than 120 kDa) could be observed in most of infected fish starting from 24 h (Fig. 6).

4. Discussion

In this study, the inflammatory response of Atlantic salmon was assessed by shotgun plasma proteomic analysis of a time-course study of an acute bacterial infection. Selected candidates were further verified by RT-qPCR and immunoassays.

The blood plasma is a highly complex mixture of proteins, where a minority of proteins constitute the majority of protein mass and the concentration difference between low abundant proteins (such as thyroid-stimulating hormone) and high abundant proteins (such as albumin) may range up to 10 orders of magnitude (Hortin and Sviridov, 2010). This dramatic concentration difference was also reflected in the proteomic analysis of salmon plasma in the present study, where abundant proteins such as serum albumin, apolipoprotein, complement factors, serotransferrin and fibronectin dominate the proteome. Thus, a substantial overlap was observed between protein identifications in individual samples, with 80% of the proteins identified in all sample groups and 37% of proteins identified in all analyzed samples. Nevertheless, we found a large number of proteins uniquely identified in infected fish, suggesting that the infection impacts the overall plasma proteome. In addition, 23 proteins which were identified in all sample

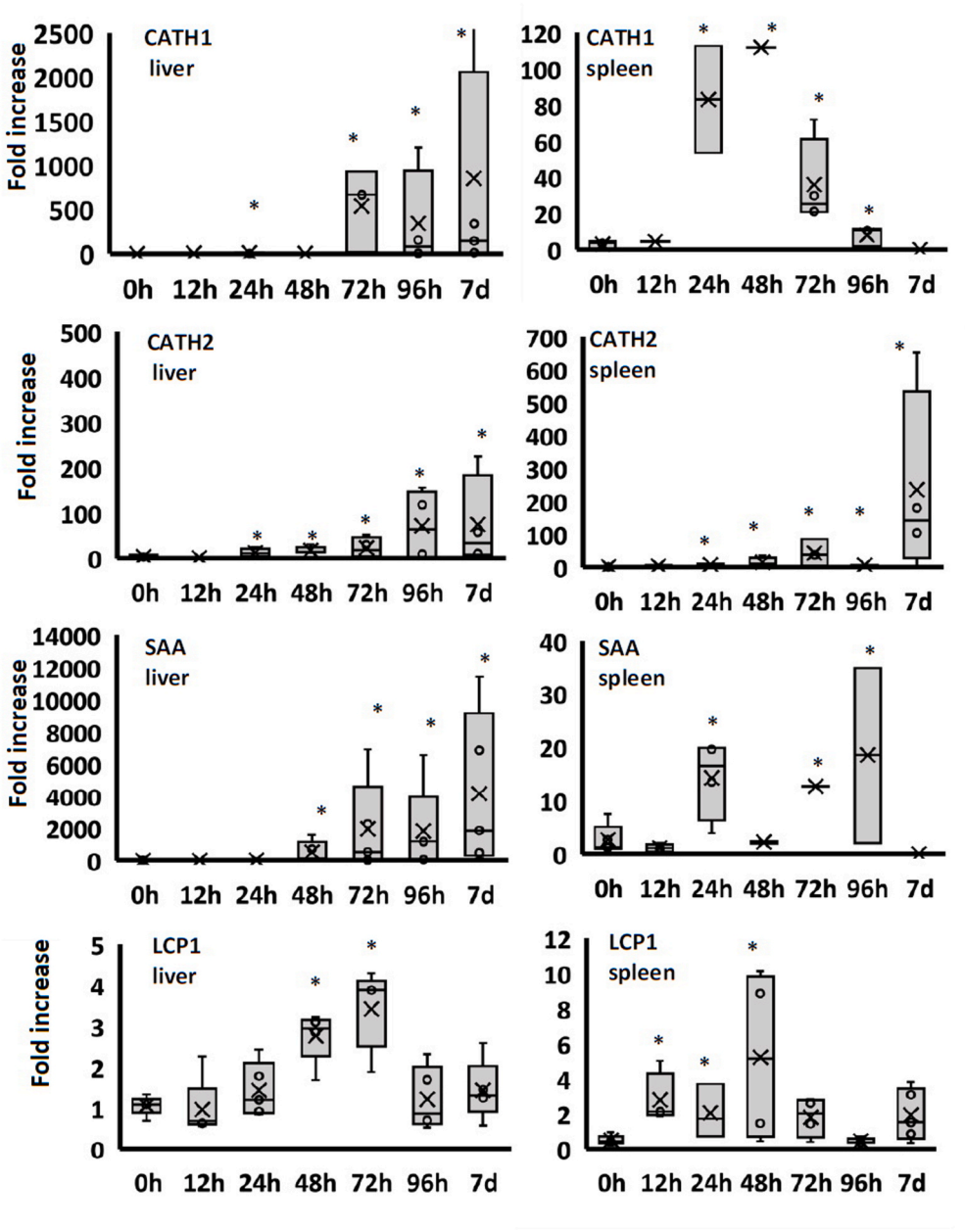

Fig. 4. Transcription of selected genes in salmon liver and spleen in infected fish at different timepoints after infection. Gene transcription was measured by RT-qPCR and is presented as fold increase (median with interquartile range) compared to control fish. Statistical significant differences ($p < 0.05$) between infected fish and control fish are indicated with an asterisk.

groups were significantly up-regulated in infected fish compared with controls.

Among the proteins identified only in infected fish, cathelicidin and L-plastin have the potential to be relevant indicators of inflammation in Atlantic salmon. Cathelicidins are initially produced as inactive prepropeptides, which consist of a signal peptide, a conserved cathelin-like domain (CLD), and a highly diversified mature peptide containing 12–80 residues (Shinnar et al., 2003). The active mature peptides undergo extracellular proteolytic processing after release from cells (Shinnar et al., 2003). Rodent cathelicidin CRAMP and human cathelicidin LL-37 are produced by many cell types, including macrophages, neutrophils, natural killer cells, and epithelial cells of the skin, intestine, airways, and ocular surface (Kahlenberg et al., 2013). Two groups of cathelicidins have been identified in salmonid fish including rainbow trout, Atlantic salmon, brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*) and grayling (*Thymallus thymallus*) (Chang et al., 2006; Scocchi et al., 2009). In salmonids, the two prepropeptides have highly similar signal peptide and cathelin-like domains, whereas the mature peptides have low similarity. In contrast to human LL37 which has α-helical structure, trout CATH1 mature peptide contains β-sheet, random coil and partial α-helical structure, while CATH2 mature peptide consists of random coils and a short piece of antiparallel β-sheet (Zhang et al., 2015). In rainbow trout, CATHs were mainly expressed by columnar epithelial cells and some lymphoid cells in gut, and lymphoid cells in head kidney, and phagocytic B cells (Zhang et al., 2015, 2017). In our proteomic results, CATHs shows up in the *salmo salar* database as several entries which represent basically the same sequences. Consistent with proteomic results, increased concentration of CATHs was shown by Western blot in blood of most bacteria infected fish from 72 h post-infection. The CATHs in blood plasma may derive from lymphoid cells including B cells (Zhang et al., 2017). Our results suggest that salmon CATH2 is a better indicator of inflammation than CATH1, due to

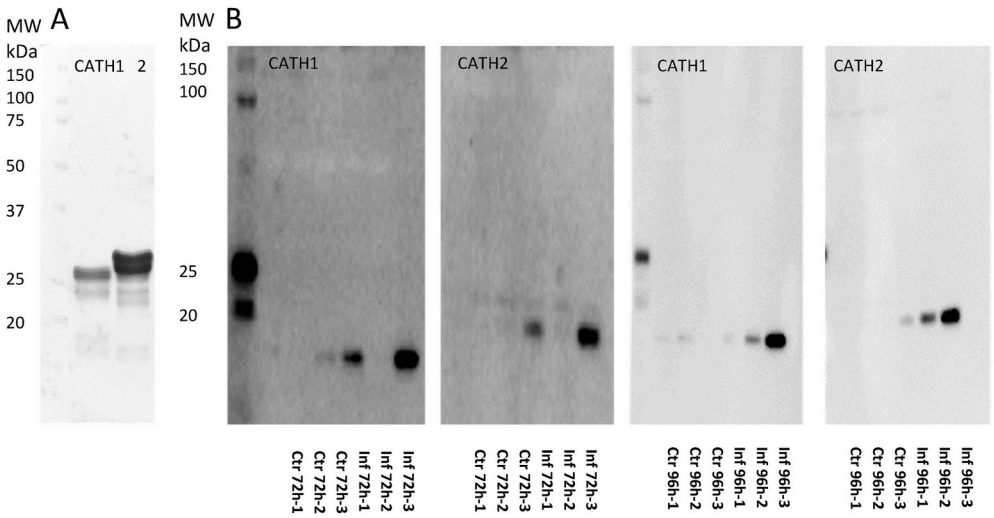

Fig. 5. A. SDS-PAGE of recombinant salmon CATH1 and CATH2 prepropeptides. **B.** Western blot of CATH1 and CATH2 in blood of control (Ctr) and bacteria infected (Inf) fish. Time point after infection and fish ID is given. Fig. 5B is one example representative of analysis using more individuals.

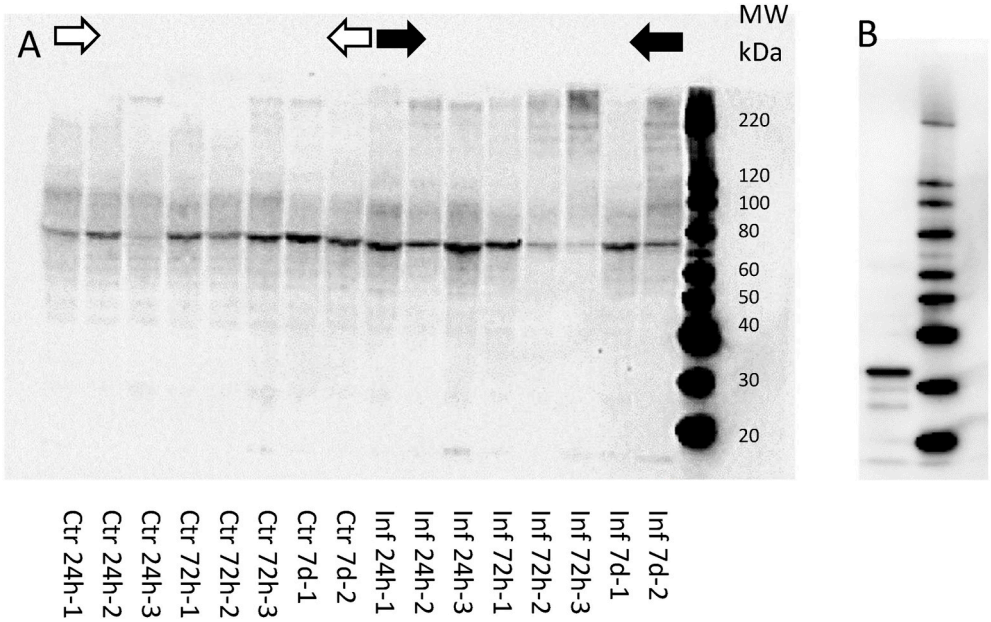

Fig. 6. A: WB of L-plastin in blood of control (Ctr) and bacteria infected (Inf) fish; **B:** WB of recombinant salmon L-plastin actin-bundling domain. Timepoint after infection and fish ID is given. Fig. 6A is one example representative of analysis using more individuals.

its absence in blood of control fish and the strong inducibility in bacteria infected fish.

L-plastin is a leukocyte-specific protein that cross-links actin filaments into tight bundles, increasing the stability of actin-based structures such as podosomes and lamellipodia (Morley, 2013). The binding of L-plastin to integrins and its localization in migrating cells, phagocytic cups, phagosomes, podosomes as well as in the immune synapse, suggest that L-plastin does not only function as an actin-bundling protein, but may be directly involved in the regulation of leukocyte adhesion and migration (Morley, 2013). L-plastin has been shown to be an abundant protein (about 1.8% of total cytoplasmic protein) in macrophages, and is used as marker of phagocytes such as neutrophils and macrophages (Pacaud and Derancourt, 1993). In the present study, L-plastin was found to be uniquely expressed in the blood of infected fish with a high number of validated proteomic spectra, strongly suggesting a role of L-plastin in the inflammatory process in salmon. This was further confirmed by Western blot. The molecular weight of L-plastin in blood is much higher than the weight of the single molecule, which may be explained by L-plastin cross-linking actin filaments or other molecules (Morley, 2013). The elevated L-plastin in blood may originate from activated phagocytes (macrophage and neutrophil) which release some components during migration or formation of extracellular traps (ETs) to trap invading bacteria (Brinkmann et al., 2004).

In the proteomic analysis, soluble TLR5 (sTLR5) was identified mainly in bacteria infected fish, but only in two control individuals. In vertebrates, TLR5 is a cell surface receptor for bacterial flagellin, a structural component of flagella and a virulence factor. The activation of this receptor mobilizes the nuclear factor NF-κB and stimulates pro-inflammatory cytokine production (Tallant et al., 2004). In teleost, in addition to the membrane-anchored TLR5 (mTLR5), the soluble variant of TLR5 has been identified in several species (Tsoi et al., 2006; Wangkahart et al., 2018). The mRNA of sTLR5 could be induced by flagellin in various tissues (Wangkahart et al., 2018), but predominantly expressed in the liver (Tsujita et al., 2004; Wangkahart et al., 2018), and

sTLR5 was more highly up-regulated than mTLR5 (Hynes et al., 2011; Wangkahart et al., 2018). In previous research, the sTLR5 was confirmed to be an acute-phase protein with integral flagellin-recognition activity and augmentation of NF-kappaB activation in rainbow trout (Tsukada et al., 2005). A two-step flagellin response against bacterial infection has been suggested in fish: flagellin first induced basal activation of NF-κB via mTLR5, facilitating the production of sTLR5 and some acute phase proteins, and secondly, sTLR5 amplifies mTLR5-mediated cellular responses in a positive feedback profile (Tsujita et al., 2004). The current proteomic result show for the first time that sTLR5 is a detectable circulating receptor in blood plasma of Atlantic salmon. In the proteomic analysis, the sTLR5 could be detected in blood of two individuals in the control group, suggesting there may be some variation of sTLR5 concentration in blood among a fish population. Further studies are needed to quantify the sTLR5 in fish at different growth stages and under infection conditions.

Among the proteins detected in all individuals, two haptoglobin-like molecules increased significantly, together with hemoglobin, in the bacteria infected fish. Some bacterial infections may cause red blood cells to rupture and release the oxygen-transporting molecule hemoglobin (Titball and Munn, 1981). As this oxidizes, it releases free heme, which can trigger programmed cell death and excessive inflammation (Larsen et al., 2010). To prevent the harmful effects of hemoglobin, haptoglobin is produced in the acute phase response to bind circulating hemoglobin. Research has also indicated that the increase of haptoglobin during inflammation is independent of the degree of hemolysis associated with the tissue injury process (Wang et al., 2001), and that any inflammatory process may increase the levels of plasma haptoglobin (Larsen et al., 2010). In fish, the elevated transcription of haptoglobin has been observed in seabream after lymphocystis disease virus (LCDV) infection and European sea bass infected with nervous necrosis virus (NNV) (Cordero et al., 2017). The concentration of haptoglobin in tilapia serum doubled at 24 h after *Aeromonas hydrophila* infection (Charlie-Silva et al., 2019), and the concentration of haptoglobin doubled in gilthead seabream blood plasma during 45 days of net handling stress (Raposo de Magalhães et al., 2020). Thus, haptoglobin is suggested to be a non-pathogen specific, universal stress marker in fish, feasible to be measured by mRNA copy numbers in tissues and by protein concentration in blood.

It is interesting to note the increased concentration of one high affinity immunoglobulin receptor Fc gamma RI (FcγR1, CD64)-like molecule in bacteria infected fish. CD64 is expressed constitutively on dendritic cells, monocytes and macrophages, and is inducible on neutrophils (Tang et al., 2018). Research in mammals show that CD64 is highly induced on the surface of neutrophils (5–10 times the normal level) within 1–6 h upon bacterial infection or inflammatory damage (Tang et al., 2018). A soluble form of CD64 (sCD64) has been identified in human blood, and the mean sCD64 levels in early rheumatoid arthritis patients correlated with monocyte CD64 expression (MFI) and the amount of IgG bound to $CD64^{+}$ cells (Matt et al., 2015). The sCD64 has been identified in several fishes. In channel catfish, sCD64 is encoded by a single-copy gene containing three Ig C2-like domains but lacking a transmembrane segment and cytoplasmic tail (Stafford et al., 2006). In addition, recombinant fish sCD64 could bind to IgM which is the predominant immunoglobulin in blood plasma (Chen et al., 2019; Stafford et al., 2006).

Compared to controls, phospholipase A2 inhibitor 31 kDa subunit-like protein was increased in infected fish. This protein is the salmon homologue of differentially regulated trout protein 1 (DRTP1) in trout (93% sequence homology). The *DRTP-1* gene encodes a protein of 88 amino acids with cysteine patterns similar with CD59. However, the DRTP-1 molecule lacks the membrane-bound C-terminal region found in CD59. The increased mRNA of *DRTP-1* have been detected in rainbow trout during the acute phase response (Talbot et al., 2009b). Furthermore, the transcription of three genes, including DRTP1, were most altered in rainbow trout after exposure to confinement stress (Talbot et al., 2009a). *DRTP1* transcription was significantly induced at 168 h after confinement stress (Pemmasani et al., 2011), and DRTP-1 microarray transcripts were increased in both liver and head kidney in Atlantic salmon infected with *A. salmonicida* (Ewart et al., 2005).

Leucine-rich alpha-2-glycoprotein (LRG1) was also increased in bacteria infected fish. LRG1 was initially identified as an inflammatory biomarker for human autoimmune diseases (Serada et al., 2010). LRG1 is a secretory type 1 acute phase protein, which is upregulated by pro-inflammatory cytokines and mainly released from liver hepatocytes. In addition, LRG1 is also stored in neutrophil granules (Druhan et al., 2017). The research of LRG1 in fish is scarce, although the LRG1 gene has been identified in several fish species (Ewart et al., 2005; Lee and Goetz, 1998). However, a recent study showed that LRG1 in blood plasma of gilthead seabream doubled under net handling stress (Raposo de Magalhães et al., 2020).

Serum amyloid A has received attention as an acute response indicator for a long time in teleost, since the transcription of *SAA* gene was shown to increase dramatically in liver after bacterial infection and in hepatocytes after proinflammatory cytokines and LPS stimulation (Goetz et al., 2004; Jensen et al., 1997; Jørgensen et al., 2000). However, in contrast to mammals, SAA has never been confirmed as an acute-phase reactant in blood of teleost fish (Villarroel et al., 2008). A recent study report increased SAA in Russian sturgeon plasma after bacterial stimulation (7.0-fold higher than controls), however the increasing SAA in plasma can only be detected in less than 50% of the challenged individuals (Castellano et al., 2020). Several studies have used proteomic approaches to study the acute-phase response in fish, but failed in demonstrating unequivocally the presence of SAA protein in the plasma of these fish (Gerwick et al., 2002). In the present study, SAA was not detected by plasma proteomics, however a SAA-specific antibody made by using soluble recombinant salmon SAA could detect positive signal in salmon plasma from a few individuals of both the infected and control groups (unpublished results). Based on the results of the current study, we suggest that SAA can only be used as acute phase reactant at the transcriptional level in Atlantic salmon.

Among the down-regulated proteins in bacteria infected fish, various fibrinogen components (including alpha, beta and gamma chains), one CD209 antigen-like protein and one CD44-like protein are involved in innate immunity. Previous research in mammals regarded fibrinogen as a "positive" acute-phase protein, i.e. its blood levels rise in response to systemic inflammation, tissue injury, various cancer and certain other events, and genetic and pharmacologic studies have unraveled pivotal roles for fibrinogen in determining the extent of local or systemic inflammation (Davalos and Akassoglou, 2012). The interface between fibrinogen and bacterial pathogens is an important determinant of the outcome of infection. First, soluble fibrinogen or fibrin matrices can physically entrap bacteria or encapsulate bacterial foci within infected tissue, thus limiting growth and dissemination of bacteria. Second, the formed fibrin can also support the recruitment and activation of host immune cells (Davalos and Akassoglou, 2012). However, in our proteomic analysis, the concentration of various fibrinogen components decreased to less than 50% in blood of *A. salmonicida* infected salmon. The reduced fibrinogen in blood may explain the hemorrhagic septicemia, which is the typical symptom of *A. salmonicida* infection. It is possible that the inhibition of the coagulation cascade by degrading fibrinogen is an invading strategy of *A. salmonicida,* induced by enzymes released from *A. salmonicida*. One such enzyme has been identified in a related bacteria, *A. sobria,* which could secret a serine protease that degrades fibrinogen and impairs human plasma clottability (Imamura et al., 2008). It is interesting to note the decreased presence of CD44 in plasma of infected fish, since CD44 is a multifunctional glycoprotein that could bind to hyaluronan, fibrin and fibrinogen (Raman et al., 2012), and CD44 glycosylation also directly controls its binding capacity to fibrin and immobilized fibrinogen (Alves et al., 2009).

Shotgun proteomic analysis of unfractionated blood samples identify a substantial amount of high-abundance proteins from blood, since the

10 most abundant proteins in plasma make up more than 90% of the protein mass. Even with such limitations, an unbiased proteomics approach was successfully applied in the present study to find proteins that were differentially expressed between bacteria infected fish and controls. The current study provides valuable protein-level evidence for the unreviewed salmon proteome. Such in-depth information obtained via LC-MS studies will undoubtedly improve our understanding of fish immune responses and the immunological variation between individuals. Following additional validation, some of these molecules (L-plastin and cathelicidins) have the potential for utilization as new biomarkers in future monitoring of health and welfare of farmed salmonids.

Author contributions

Conceptualization, BS, PB, HH, HL; methodology, BS, DvD, IM, HH; software, DvD, HH; writing—original draft preparation, BS, HH; writing and revision—all authors; projec0074 administration, HL; funding acquisition, HL. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104268.

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