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Application of 4 × 44 Oligo Microarray to Transcriptomic Analysis of Immune Response in Rainbow Trout Infected with *Aeromonas salmonicida*

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Abstract: Rainbow trout, one of the most economically important aquaculture fish species worldwide, is affected by the pathogenic bacteria *A. salmonicida*, which causes furunculosis outbreaks, leading to huge economic losses. In this study, an oligonucleotide microarray was applied to identify transcriptional changes in the skin of rainbow trout individuals in response to a bacterial infection. Overall, 656 and 434 differentially expressed genes (DEGs) were identified at 2 and 6 days after a bacterial challenge (dpi), respectively. A comparison of moribund (2 dpi) and survivor fish (6 dpi) revealed 169 DEGs. Between these were many genes involved in immune response, including lysozymes, pattern recognition receptors (c-type lectins), antimicrobial peptides (cathelicidin and hepcidin), acute-phase proteins (serum amyloids and haptoglobin), complement cascade proteins (*c3*, *c4*, *c6* and *c7*), interleukins (*il11* and *il1b*) and chemokines (*ccl19* and *cxcl8*). Alterations of leptin, eicosanoids and prostaglandins have been found, which suggest metabolic remodeling in conjunction with immune response. Further, the regulation of programmed cell death genes (caspase 8, bcl2 apoptosis regulator, nfkb inhibitor alpha and heme oxygenase) and structural proteins (collagens, myosins, keratins and metalloproteinases) was observed. This study provides, for the first time, a gene expression analysis of rainbow trout skin in response to *A. salmonicida* infection, revealing the complexity of defense strategies in response to furunculosis.

Keywords: microarray; rainbow trout; *Aeromonas salmonicida*; furunculosis; infection; skin

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1. Introduction

Farmed fish such as rainbow trout (*Oncorhynchus mykiss*), which is one of the major aquaculture species, with global production reaching about 952 thousand tons of live weight in 2021 [1], must cope with multiple stressors (hypoxia, temperature and crowding) in an intensive aquaculture environment. Exposure to a stressful environment enhances the spread of pathogenic bacteria and causes disease outbreaks [2]. Infectious diseases caused by bacterial pathogens lead to the high mortality of fish in aquaculture populations and significant economic losses. One of the bacteria that affect salmonids, including rainbow trout, is a Gram-negative, non-motile and facultative anaerobic bacteria called *Aeromonas salmonicida* spp. *salmonicida* (*A. salmonicida*). The histopathology of *A. salmonicida*-infected rainbow trout includes inflammatory lesions in the dermis, kidney and liver, vascular congestion and the cytoplasmic vacuolization of hepatocytes [3]. The major virulence factor of *A. salmonicida* is a type three secretion system (TTSS). A TTSS functions by moving bacterial effector proteins to the cytosol of the host, affecting the immune system [4]. After host penetration, bacterial effector

molecules are able to modulate and disrupt the cytoskeleton and cell-signaling cascades and may induce apoptosis [5]. Besides salmonids, this bacterium can also infect other fish species such as cod (*Gadus morhua*) [6], common carp (*Cyprinus carpio*) [7], seabream (*Sparus aurata*) [8], sea bass (*Dicentrarchus labrax*) [9], senegalese sole (*Solea senegalensis*) [10], halibut (*Hippoglosus hippoglosus*) [11], sea lamprey (*Petromyzon marinus*) [12] and turbot (*Scophthalmus maximus*) [13]. Infection by *A. salmonicida* causes furunculosis. The disease begins with epithelial hyperplasia, followed by furuncles, lesions and hemorrhages of the skin and muscles and darkening of the skin, and it finally leads to septicemia and fish death [14,15]. Furunculosis is transmitted via water and through direct contact between infected and healthy fish, and one of the signs of infection may be lethargic swimming and a loss of appetite [16]. Since the disease spread depends on temperature (the optimum is from 12.8 to 21.1 °C), it is expected that climate changes manifested by rising water temperatures may increase the susceptibility to the disease in aquaculture and wild fish populations [17,18].

Aquaculture global production reached over 126 million tons in live weight in 2021 [1], and according to the Food and Agriculture Organization (FAO) of the United Nations report from 2022, it will rise in the future [19]. Because aquaculture is currently the fastest growing food sector in the world, the development of effective treatments against pathogens such as *A. salmonicida* is very important. Despite best efforts to control furunculosis, it still poses a serious threat to salmonids, and outbreaks are common [18]; thus, new antibacterial treatments need to be found.

Previously, the response to *A. salmonicida* has been studied in rainbow trout through a gene expression analysis in different tissues such as the gills [20,21], head kidney [22], liver [21,23] and spleen [21,24], but not in the skin. The transcriptome response to *A. salmonicida* infection has also been investigated in other fish species, such as Atlantic salmon (*Salmo salar*) [25,26], cod [27], turbot [28] and lumpfish (*Cyclopterus lumpus*) [29]. More information is required since fish have shown divergent susceptibility to bacterial infection, and even closely related species such as Atlantic salmon, brook trout (*Salvelinus fontinalis*) and rainbow trout showed intra-specific resistance to pathogens [30–32]. This study applied a 4 × 44 oligonucleotide microarray to investigate the response of rainbow trout to an infection with a pathogen strain of *A. salmonicida.* Gene expression changes were studied in the fish skin, which is a barrier to infection. Despite many studies on the rainbow trout response to *A. salmonicida*, gene expression in the skin and adhering tissues including the skeletal muscles has not been analyzed.

2. Materials and Methods

2.1. Ethics Statement and Experiment Description

Experimental procedures were performed in accordance with the three Rs for the humane use of animals in scientific research and were approved by the Local Ethics Committee on Animal Experimentation of the Inland Fisheries Institute of Olsztyn, Poland (Nr 20/2011). Rainbow trout were obtained from the Department of Salmonid Fish Research, Inland Fishery Institute, Rutki, Poland. Fish were 1 year old, with an average length of 155 mm and an average weight of 50 g. The conditions of fish were inspected prior to experiment, including checking for the presence of pathogens. *Polymerase chain reaction* (PCR) did not reveal the presence of the following viruses: viral hemorrhagic septicemia (VHS) [33], infectious haematopoietic and pancreatic necrosis (IHN and IPN) [33–35] and salmonid herpesviruses [36]. Biological methods (API 20E test and growth medium) did not reveal the presence of *A. salmonicida*. All fish were in good condition, and no changes indicative of ongoing disease process were observed in the fish prior to the experiment. Fish were kept in plexiglass tanks with fresh water and temperature of about 15 °C, and they were fed twice daily with commercial pellets [37]. The infection experiment was carried out in the Department of Fish Pathology and Immunology, Inland Fisheries Institute (Żabieniec, Poland), according to their developed procedure, as follows [37]: Pathogenic bacteria, *A. salmonicida* spp. *salmonicida*, after growing on a solid support and washing,

were cultured. The fish were infected via intraperitoneal injection [38,39] around the left pectoral fin with *A. salmonicida* bacteria diluted in phosphate-buffered saline (PBS) to a concentration of 1×10^7 colony-forming unit (CFU) mL^{-1}, 0.2 mL per fish [37]. Of the infected fish, four (RT1, RT2, RT3 and RT4) were sampled up to 2 days post infection (2 dpi), and these fish were moribund. Three other fish (RT5, RT6 and RT7) were collected 6 days post infection (6 dpi), and these fish were survivors. Further, three healthy, uninfected fish (control) were kept in a separate tank in the same conditions as described above. Control fish were injected with sterile PBS [37] (Siwicki A., personal communication) and sampled after six days of experiment. Propiscin was used to anaesthetize the studied fish [40].

2.2. Immunoassay Analysis

Blood from all fish used in this study was sampled for immunoassay analysis and centrifuged for 10 min at 4 °C with 8500 revolutions per minute (rpm) [41]. Immunological tests included determining the following parameters of non-specific humoral immunity: lysozyme and ceruloplasmin activity in the plasma and the levels of gamma globulin in the serum. Lysozyme activity was measured with the turbidimetric method using a *Micrococcus lysodeicticus* suspension in a sodium phosphate buffer and the standard egg white lysozyme (both Sigma-Aldrich, Saint Louis, MO, USA) according to Siwicki and Anderson [42]. Ceruloplasmin activity was measured with the spectrophotometry method in an enzyme reaction mixture containing 0.2% p-phenylenediamine (PPD) in acetate buffer and 0.02% sodium azide solution according to Siwicki and Anderson [42]. The optical density was read at 540 nm. Gamma globulin was measured using modification of the Lowry micro-method, presented by Siwicki and Anderson [42], depending on the precipitation of the total immunoglobulin with polyethylene glycol (10,000 kDa; Sigma-Aldrich, Saint Louis, MO, USA) and centrifugation to separate bound immunoglobulin fraction from the supernatant.

2.3. RNA Extraction

Samples of skin with adhering skeletal muscles (1 cm × 0.2 cm) from the area of the anus, where the ulcers showed up, were collected with the aid of a scalpel and washed in sterile diethyl pyrocarbonate (DEPC) water. Total RNA was extracted using the GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Saint Louis, MO, USA). The isolated RNA samples were diluted to 5 ng/µL in DEPC water and stored at −70°C. Concentration of isolated RNA was determined by measuring absorbance at 260 nm using the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) [15]. Integrity of RNA was checked by using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and RNA Integrity Number (RIN) > 7.5 was accepted.

2.4. Microarray Analysis

Two-color microarray analysis was performed with uninfected (n = 3) and infected fish (n = 7), labeled with Cy3 and Cy5 dyes, respectively, using the Two-Color Low Input Quick Amp Labeling kit (Agilent, Santa Clara, CA, USA). The hybridization was performed in the Department of Physiological Sciences, Warsaw University of Life Sciences (SGGW) with the Agilent-028639 RTIQ custom-commercial 4 × 44 K oligo microarray (GEO accession no. GPL16819; Agilent, Santa Clara, CA, USA) using Gene Expression Hybridization Kit (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. This microarray consisted of 43,509 salmonid and 60 mer oligonucleotides, and its preparation was in accordance with a previously developed protocol [43]. The hybridized arrays were washed using the Agilent Gene Expression Wash Buffer Kit and scanned in an Agilent Technologies Scanner G2505C according to the manufacturer's protocol (GE2_1010_Sep10). The scanned microarray images were analyzed using the Agilent Feature Extraction software (version 10.10.1.1) [44]. The raw mean signal was background-corrected using the BackgroundCorrect function (normexp method) and next normalized within and between microarrays (lowess and quantile normalization, respectively) in the limma package in R [44,45]. All

differentially expressed genes (DEGs) were identified using limma (fit linear model) and confirmed with the RankProd package (fold-change (FC) ≥ 2 and p-value < 0.05) [45,46].

2.5. Functional Annotation

DEGs were searched against proteins from the NCBI non-redundant (nr) database using the Basic Local Alignment Search Tool (BLASTX) implemented in BLAST+ (v.2.2.29) [47], with an E-value threshold of 10^{-5}, using sequences from which probes were designed. For functional annotation, gene ontology (GO) terms were assigned to the DEGs using Blast2GO software (version 6.0.3) [48] with the same E-value. Further, gene symbols were assigned to each DEG using the Zebrafish Information Network (ZFIN) and the HUGO Gene Nomenclature Committee (HGNC) databases. GO and pathway enrichment analyses were performed using *Kyoto Encyclopedia of Genes and Genomes* (KEGG) Orthology-Based Annotation System (KOBAS v.3.0) for the human data (corrected p-value < 0.05) [49].

2.6. RT-qPCR Validation

To verify the microarray results, 10 DEGs were screened via RT-qPCR (Table 1). The primers were designed using Primer3 software, version 0.4.0 [50], and eukaryotic translation elongation factor 1 alpha (*eef1a*) was used as a reference gene. Sequences of the primers were analyzed for hairpin structure and self- and hetero-dimers in Vector NTI Express software (v1.1.1). Same biological RNA samples were used for RT-qPCR analysis, including uninfected fish (n = 3), moribund (n = 4) and survivors (n = 3). Annealing temperature was optimized for each primer pair. PCR reactions were performed using SensiFASTTM SYBR No-ROX One-Step Kit (Bioline, Memphis, TN, USA). The PCR Master Mix included 400 nM concentration of forward and reverse primers and 10 ng per reaction of RNA sample. Reactions were performed in Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). The standard cycling conditions were as follows: reverse transcription at 45 °C for 10 min and 95 °C for 2 min for polymerase activation, followed by 45 cycles of denaturation, annealing and extension. Melting curve analysis was conducted to confirm that primers did not form primer dimers and non-specific amplification product did not appear. Gene levels were calculated using the $2^{-\Delta\Delta Ct}$ method [51].

Table 1. Primers used for RT-qPCR.

Microarray SPOT_ID	Gene	Primers (5′-3′)	Product Size (bp)
Omy#S27585481	Serum amyloid a1 (*saa1*)	F: GGAAGCTGGTAGTGGTTCAC R: TGTACTCCTCGTTATCCATG	100
Omy#S26387020	Cathelicidin antimicrobial peptide *(camp*)	F: GTATGAAGACATCATCACAG R: CATCCTCTGTATTCAAAGTC	110
Omy#S15341081	Prostaglandin-endoperoxide synthase 2 (*ptgs2*)	F: TCAACAACTCCCTGGTCAC R: GAGGCAGGTTCCGTCCAC	99
Omy#S34308694	Steap4 metalloreductase (*steap4*)	F: CAACAGGCTTCCCTTTCATC R: GCATCCACACAAACAACCAG	108
Omy#S15301030	Matrix metallopeptidase 13 (*mmp13*)	F: GGACCAGGAGACAGTTACGC R: CATTCATTGTTGTTCATGGC	106
Omy#S16761102	Interleukin 17D (*il17d*)	F: TTCGTGTCCAACAGAAGTGC R: GACACCTTGGCTACCGATGC	99
Omy#S15341279	Mx dynamin like gtpase 1 (*mx1*)	F: GGCAGAGAGGCTGTATTTCC R: TGAGACGAACTCCGCTTTTC	101
Omy#S18101422	Cathepsin L (*ctsl*)	F: GGAAGCTGGTAGTGGTTCAC R: TGTACTCCTCGTTATCCATG	99
Omy#S18164841	C-C motif chemokine ligand 13 (*ccl13*)	F: CCATGAAGACCCTGACTGC R: TCCTCGGGCTGAACTTTAG	120
Omy#S15340857	Cathepsin B (*ctsb*)	F: AGAACTTCCACAATGTTGAC R: CTGGCAGACTCATGTCCTC	111

2.7. Data Availability

The microarray data were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (NCBI GEO) under the accession number GEO: GSE230658.

3. Results

3.1. Bacteriological and Immunoassay Analysis

A bacteriological analysis confirmed the presence of the *A. salmonicida* pathogenic strain in the fish after the intraperitoneal injection. An analysis of the blood of the infected fish in comparison with the control revealed that the ceruloplasmin (Cp) activity decreased after the infection (p-value < 0.05) in both the moribund and survivor groups, and there was no statistically significant difference between 2 dpi and 6 dpi (Figure 1a). The level of gamma globulin (Ig) decreased after 2 days of infection (p-value < 0.05) and then increased in the survivor fish (6 dpi) to the control level (Figure 1b). On the other hand, the lysozyme activity increased within 2 days post infection (p-value < 0.05) and then decreased to the control level after 6 days (Figure 1c).

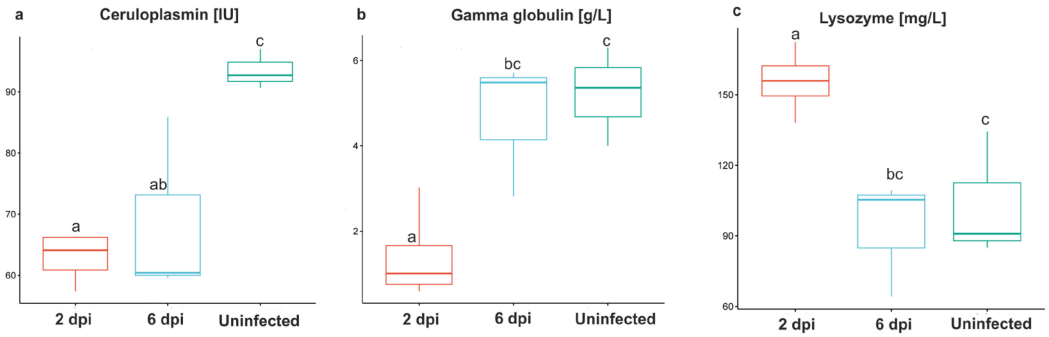

Figure 1. Immunoassay analysis of infected fish: 2 dpi (moribund, n = 4), 6 dpi (survivors, n = 3) and uninfected (control, n = 3). Statistical comparison was carried out using Kruskal–Wallis test. (**a**) Ceruloplasmin activity in the plasma. (**b**) Gamma globulin level in the serum. (**c**) Lysozyme activity in the plasma. Significant differences are indicated by different lowercase letters (p-value < 0.05); same letter indicates no statistical difference.

3.2. Microarray Analysis

The Pearson correlation of all genes showed that the gene expression profile was similar through all of the rainbow trout specimens used in the experiment (average R = 0.71, p-value < 0.05; Figure 2).

In this study, the limma and RankProd packages in R were used to identify the DEGs among the samples (FC > 2, p-value < 0.05; Supplementary Table S1). In total, 656 and 434 genes showed statistically different expressions after 2 and 6 days of infection, respectively (Figure 3a,b). Of these, 382 and 221 were up-regulated, and 274 and 213 were down-regulated at 2 dpi and 6 dpi, respectively (Figure 3a,b). On average, 98.05% of the DEGs were annotated using NCBI nr databases (excluding uncharacterized, unnamed and hypothetical proteins), and gene symbols using Zfin and HGNC databases were assigned to 92.58% of them. Further, using Blast2GO software (version 6.0.3), gene ontology (GO) terms were assigned to 76,58% of the annotated DEGs and they were classified into three main categories: biological processes, molecular function and cellular component (Supplementary Table S1). In the moribund group, the highly expressed genes belonged to the cytokine–cytokine receptor interaction pathway, such as *cd209*, the c-type lectin domain family 4 member e (*clec4e*), interleukin 11 (*il11*) and potassium channel tetramerization domain-containing 12 gene (*kctd12*), which is involved in ion transport. In the fish that survived, the highly expressed genes were related to lipid metabolism, such as mid1 interacting protein 1 (*mid1ip1*) and matrix metallopeptidase 13 (*mmp13*), which is involved in collagen degradation. In both groups, a gene encoding calcium-binding protein and

coiled-coil domain 1 (*calcoco1*) were highly increased (Figure 3a,b). On the other hand, in the 2 dpi group, the down-regulated genes were the immune-related c-c motif chemokine ligand 13 (*ccl13*), liver enriched antimicrobial peptide 2 (*leap2*) and c1q and tnf-related 3 (*ctrp3*). In the 6 dpi group, the genes with the lowest expressions were s100 calcium-binding protein p (*s100p*), cathepsin (*ctsl1*) and MHC class I heavy chain. In both groups, the heavily reduced genes were heat shock protein 90 alpha family class a member 1 (*hsp90aa1*) and inositol 1,4,5-trisphosphate receptor type 2 (*itpr2*), which is involved in cell cycle and calcium transport (Figure 3a,b).

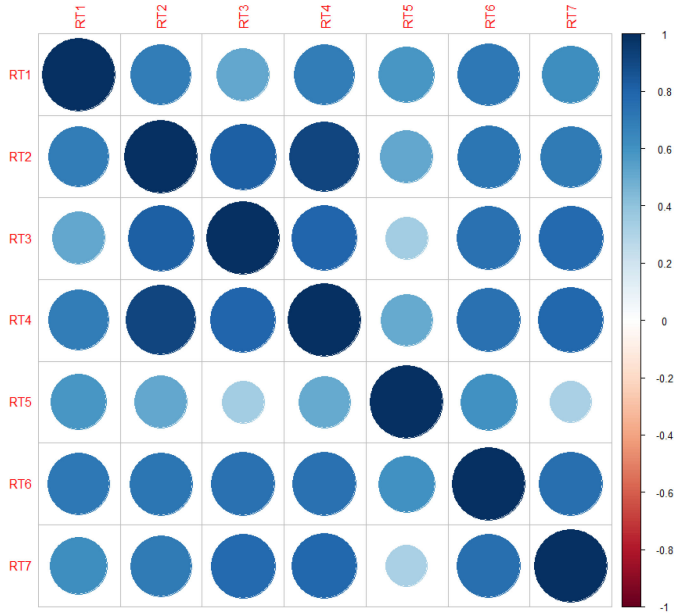

Figure 2. Pearson correlation coefficient analysis between infected fish, presented as matrix graphic (RT1, RT2, RT3 and RT4 were moribund, whereas RT5, RT6 and RT7 were survivor fish). All samples showed positive correlation. Color and size of circles (light blue to dark blue) indicate rising correlation value.

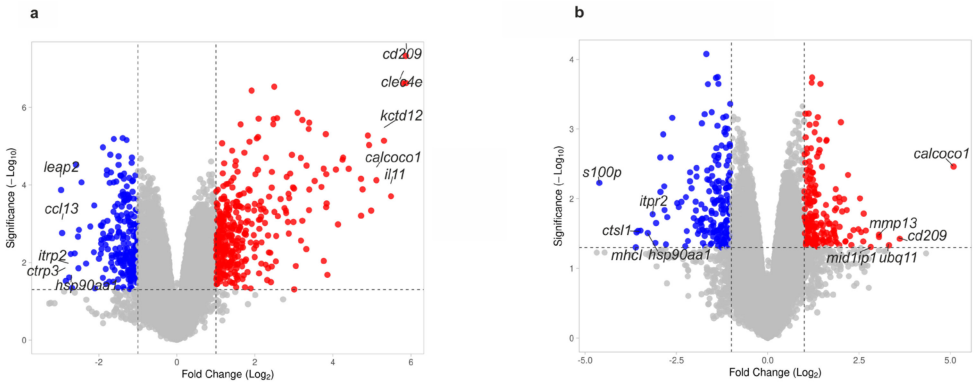

Figure 3. Volcano map of differentially expressed genes (DEGs) in (**a**) moribund fish (2 dpi group) and (**b**) survivor fish (6 dpi group). Red color represents up-regulated genes, whereas blue color shows down-regulated genes. Grey color shows non-significant genes. Differentially expressed genes were identified based on fold change (FC) > 2 (FC($\log_2$) > 1 on *x*-axis) and significance($-\log_{10}$) over 1.3 (*y*-axis), which represents *p*-value < 0.05. Gene symbols are presented for top five DEGs with highest and lowest expression values in each experimental group. Gene symbols: liver-enriched antimicrobial peptide 2 (*leap2*), c-c motif chemokine ligand 13 (*ccl13*), inositol 1,4,5-trisphosphate receptor type 2 (*itpr2*), c1q and tnf-related 3 (*ctrp3*), heat shock protein 90 alpha family class a member 1 (*hsp90aa1*), cd209 molecule (*cd209*), c-type lectin domain family 4 member e (*clec4e*), ion transport

potassium channel tetramerization domain containing 12 gene (*kctd1*), interleukin 11 (*il11*), calcium binding and coiled-coil domain 1 (*calcoco1*), s100 calcium binding protein p (*s100p*), cathepsin (*ctsl1*), MHC class I heavy chain (*mhcI*), mid1 interacting protein 1 (*mid1ip1*), matrix metallopeptidase 13 (*mmp13*) and polyubiquitin 11 (*ubq11*).

A comparison between the moribund (2 dpi) and survivor groups (6 dpi) using a Venn diagram revealed that only 24.71% and 22.06% of the gene symbols and probe names were shared between the groups (Figure 4a,b).

Moreover, the Wilcoxon rank sum test using the absolute value of the $\log_2$FC revealed that the median expression was significantly different between the studied groups, and it was greater in the moribund fish compared to the survivor fish (p-value $< 2.2 \times 10^{-16}$).

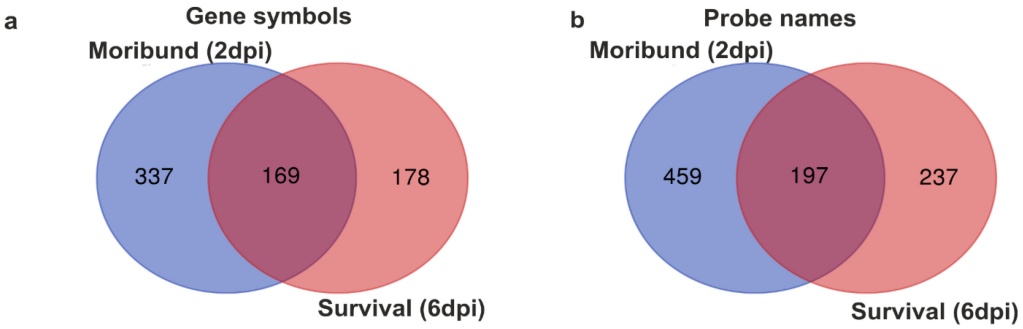

Figure 4. (**a**) Venn diagram of shared gene symbols between moribund and survivor fish (2 dpi vs. 6 dpi). (**b**) Venn diagram of shared probe names between moribund and survivor fish (2 dpi vs. 6 dpi).

Based on gene symbols, a pathway and GO enrichment analysis was performed (Figure 5a,b; Supplementary Figures S1 and S2; p-value < 0.001). A comparison between the identification numbers (IDs) revealed that 27.93% of the up-regulated pathways and 32.83% of the down-regulated pathways were shared between the moribund and survivor groups. The obtained results revealed that an infection with *A. salmonicida* increased the immune system response (c-type lectin receptor signaling pathway, cytokine–cytokine receptor interaction, il-17 signaling pathway and neutrophil degranulation), apoptosis, autophagy and collagen degradation in both the moribund and survivor fish. Further, the cellular senescence, chemokine signaling pathway, complement and coagulation cascades, ferroptosis, fc gamma r-mediated phagocytosis and the foxo signaling pathway were uniquely enriched at 2 dpi. After 6 days post infection, the fatty acid metabolism, DNA repair, programmed cell death, MHC class II antigen presentation, intrinsic pathway for apoptosis and the citric acid (TCA) cycle and respiratory electron transport were enriched. The pathways involved in collagen degradation and formation, glycolysis/gluconeogenesis, extracellular matrix organization and protein digestion and absorption were decreased in both of the infected groups. Further, in the 2 dpi group, genes involved with the cell cycle, DNA replication and ECM receptor interaction were down-regulated, whereas apoptosis, vitamin digestion and absorption, glycine, serine and threonine metabolisms and ion channel transport were decreased in 6 dpi.

To identify the differentially expressed genes between groups, the limma package was used. An analysis revealed 169 DEGs, of which 76 increased over time and 93 decreased over time (Figure 6a, Supplementary Table S2). To present the relationship between the samples, hierarchical clustering was carried out using all of the identified genes (169 DEGs; Figure 6b). Genes such as cerebellin 1 (*cbln1*; FC = 19.26), complement c1q-like protein 2 (*c1ql2*; FC = 17.49), proteasome subunit beta type-7 (*psmb7*; FC = 17.22), c-type lectin domain family 4 member e (*clec4e*; FC = 17.21) and proteasome subunit beta type-8 (*psmb8*; FC = 8.42) significantly decreased over time. On the other hand, the expressions of c1q and tnf-related 3 (*ctrp3*; FC= 5.03), interferon-inducible protein gig2 (*gig2p*; FC = 4.92), myelin

and lymphocyte protein-like (*mal*; FC = 4.52), cytochrome c (*cyc*; FC = 4.35) and keratin type I cytoskeletal 13 (*krt13*; FC = 3.63; Supplementary Table S2) increased over time.

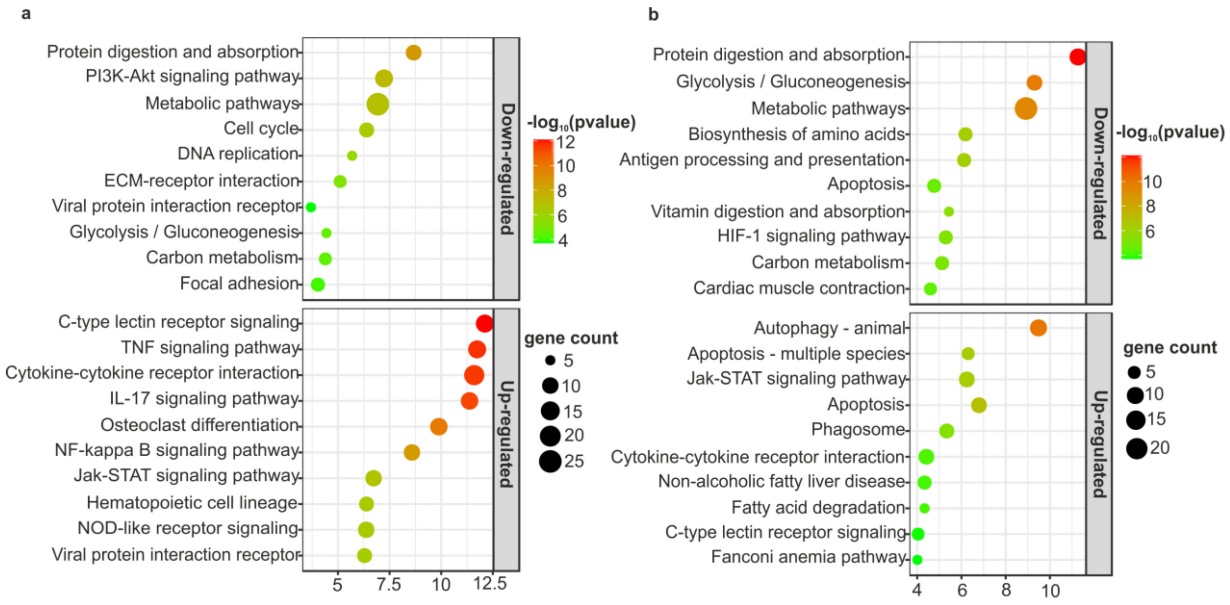

Figure 5. Bubble plot of pathway enrichment analysis using KEGG database. Plot presents top 10 KO subcategories with highest *p*-value. (**a**) Moribund fish (2 dpi). (**b**) Survivor fish (6 dpi).

The KEGG orthology (ko) and GO enrichment analysis revealed that the expression of genes involved in the inflammatory response, il-17 signaling pathway, c-type lectin receptor signaling, tnf signaling and NF-kappa B signaling pathway decreased over time, whereas genes involved in oxidative phosphorylation, fatty acid metabolism, the citric acid (TCA) cycle and respiratory electron transport and mineral absorption increased over time (Figure 7 and Supplementary Figure S3).

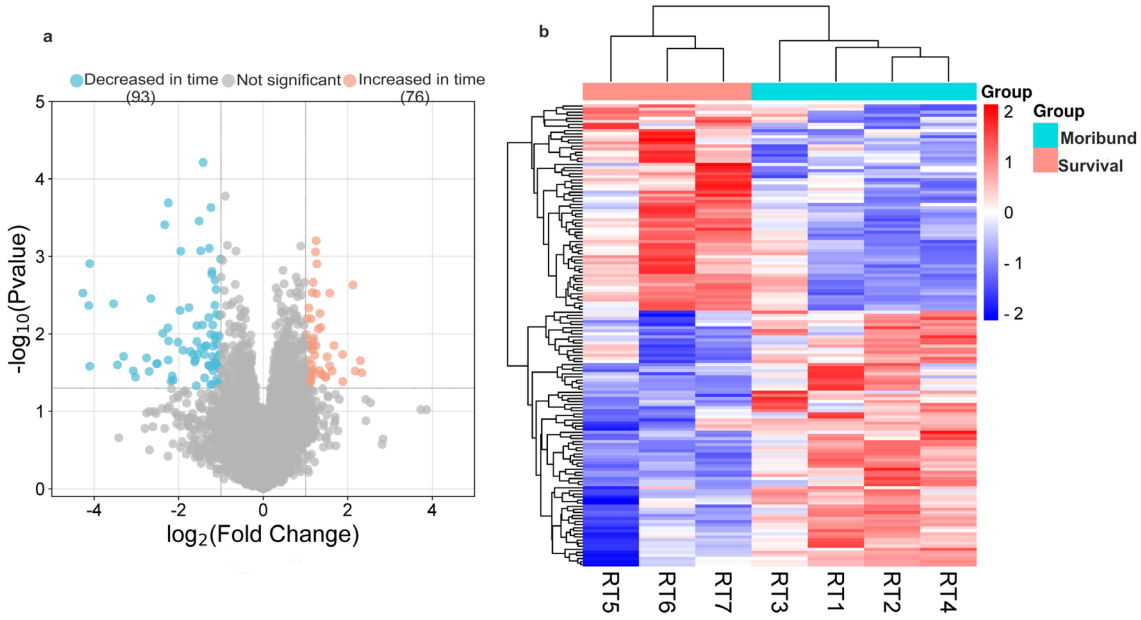

Figure 6. (**a**) Volcano map of differentially expressed genes (FC > 2; *p*-value < 0.05). (**b**) Hierarchical clustering of 7 infected fish using identified DEGs. Column represents infected individuals, and row represents a gene.

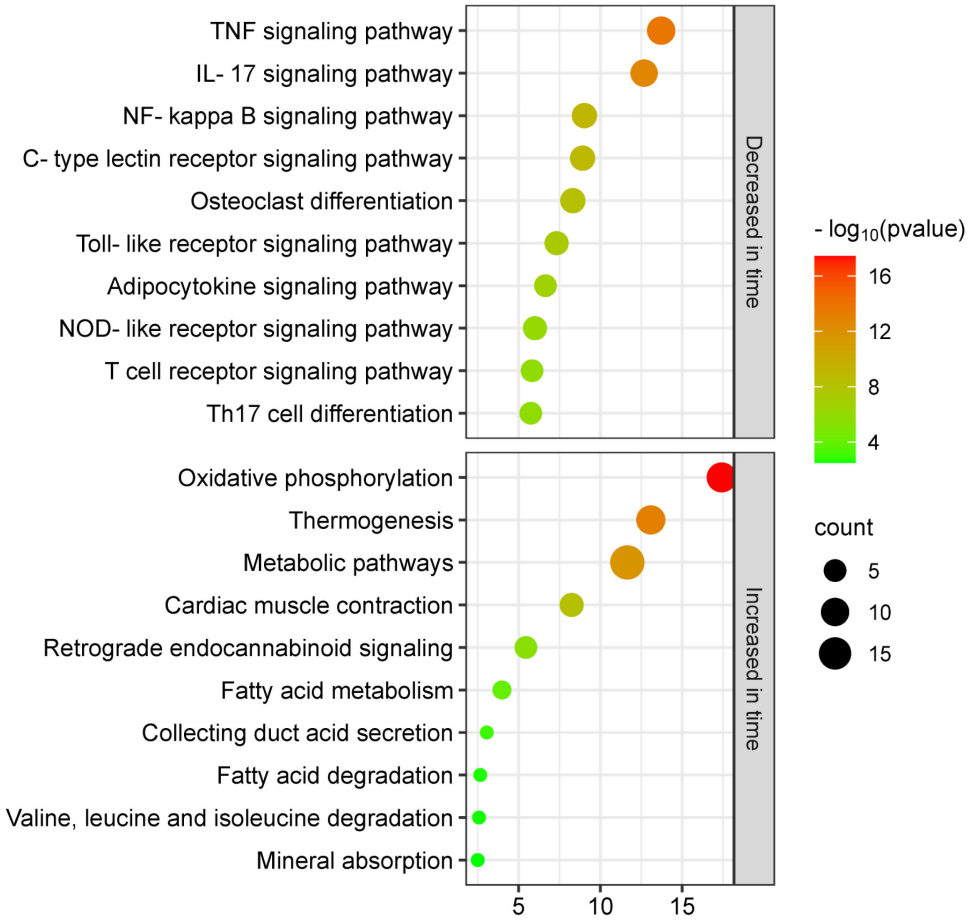

Figure 7. Bubble plot of pathway enrichment analysis using KEGG database of 169 DEGs. Plot presents top 10 pathways with highest *p*-value that decreased and increased over time.

3.3. RT-qPCR Validation

Expression of ten selected genes related to immune response, apoptosis, extracellular matrix organization and metabolism, including serum amyloid A1 (*saa1*), cathelicidin antimicrobial peptide (*camp*), prostaglandin-endoperoxide synthase 2 (*ptgs2*), steap4 metalloreductase (*steap4*), *mmp13*, interleukin 17d (*il17d*), mx dynamin-like gtpase 1 (*mx1*), cathepsin L and B (*ctsl* and *ctsb*) and *ccl13* were verified via RT-qPCR (Figure 8a). The expression trends of these genes were significantly correlated with the microarray results (R = 0.98 and R = 0.97; Figure 8b,c). These results confirmed the reliability of the microarray analysis.

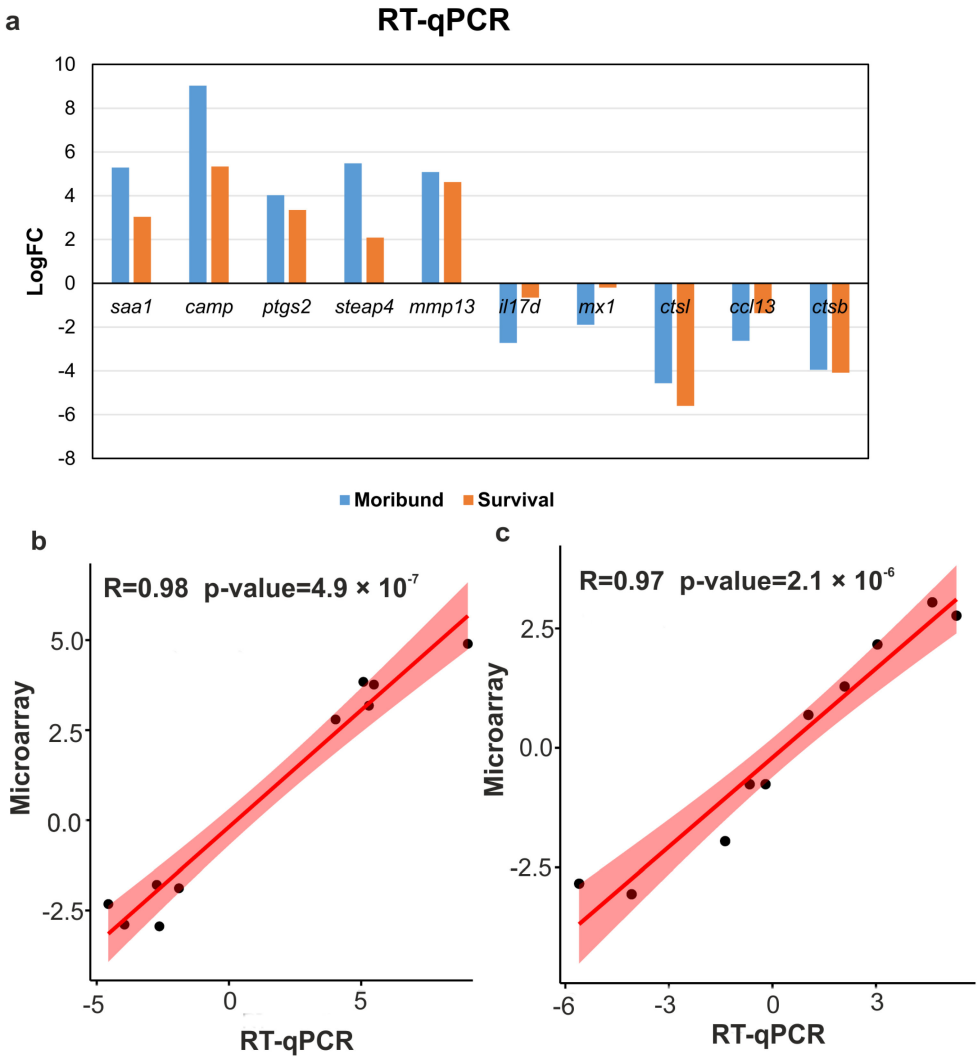

Figure 8. (**a**) RT-qPCR results for moribund (blue) and survivor (orange) fish; (**b**) Pearson correlation between microarray and RT-qPCR expression levels for moribund fish (2 dpi); (**c**) Pearson correlation between microarray and RT-qPCR expression levels for survivor fish (6 dpi). Red line represents a best fit line (with confidence intervals around the slope).

4. Discussion

Farmed fish are commonly exposed to many pathogens in intensive systems. Bacterial diseases, such as furunculosis, are a major concern for aquaculture due to heavy economic losses [52]. Fish skin is a multifunctional organ that protects the organism from the environment and is the first barrier against infection. One of the first symptoms of furunculosis is the darkening of the skin and the emergence of ulcers, which lead to sepsis and fish death. Despite massive vaccination, this disease is still a threat for fish, which can worsen because temperature increases due to climate change will promote *A. salmonicida* infection [18]. In the present study, we applied a 4 × 44 oligonucleotide microarray to investigate the gene expression in the skin with adhering skeletal muscles of the rainbow trout subjected to *A. salmonicida* infection. Further, the gene expression results obtained using the microarray method were validated via a RT-qPCR analysis of the selected genes. The results of the mRNA quantification using these two methods were consistent.

Our studies showed that in both the moribund and survivor fish (2 and 6 dpi), a bacterial infection caused the down-regulation of several genes involved in the glycolysis/gluconeogenesis pathway, such as aldolase and fructose-bisphosphate (*aldoc* and *aldob*), bisphosphoglycerate mutase (*bpgm*), phosphofructokinase, muscle (*pfkm*) and phos-

phoglycerate kinase 1 (*pgk1*). In both groups, the expression of the leptin (*lep*) gene was raised, which is involved in the regulation of food intake and body fat and exerts powerful peripheral modulations on immune cells; thus, it may participate in the interaction between the immune response and metabolism [23,53]. Since a bacterial infection affects the glycolysis/gluconeogenesis, leptin changes the fuel source to fatty acids (lipid) [54], and this shift might protect them from sepsis [55]. Up-regulated phospholipase A2 (*pla2g12a* and *pla2g4a*) and 5-lipoxygenase activating protein (*alox5ap*) are involved in biosynthesis eicosanoids, which are lipid-derived mediators of inflammation. Other genes involved in eicosanoids metabolism and lipid peroxidation such as prostaglandin-endoperoxide synthase 2 (*ptgs2*), 15-hydroxyprostaglandin dehydrogenase (*hpgd*) and prostaglandin reductase 1 (*ptgr1*) were differentially expressed between the moribund and survivor fish. The expression of these genes was higher in the first 2 days post infection and decreased in time to the control level (p-value < 0.05). Previously, the role of *ptgs2* has been studied in the immune response against *Aeromonas hydrophila* infection in common carp [56]. In summary, in accordance with previous studies in vertebrates [57,58], these results indicate metabolic reprogramming in conjunction with the immune response in rainbow trout infected by *A. salmonicida*. Lysozyme is an important mucosal antibacterial enzyme that lyses pathogens. In this study, two types of lysozymes (c-type and g-type) were overexpressed in the skin of rainbow trout after 2 days of infection and then decreased in the next days; however, only *lysozyme c* showed a statistically significant difference. Further, the lysozyme activity in the blood also increased at 2 dpi compared to the uninfected fish, and then it decreased at 6 dpi to the control level, which additionally confirms the activation of the innate humoral system up to 2 days post infection. Lysozyme was decreased in the head kidney of rainbow trout stimulated with *A. salmonicida* [23]. On the other hand, the up-regulation of lysozyme was detected in the skin tissue of crucian carp (*Carassius auratus*) in response to *Aeromonas hydrophila* [59]. Both up- and down-regulation of lysozyme was observed in the gill tissue of rainbow trout depending on the transcript variant [20]. Thus, the expression profile of lysozymes after an infection with *A. salmonicida* depends on the fish species, type of tissue and time after infection.

The first line of defense against pathogens constitute pattern recognition receptors (PRRs) that are innate immune sensors responding to conserved patterns of microorganisms [60]. In this study, several c-type lectin receptors (CLR) showed up-regulation after *A. salmonicida* infection, and some of them, such as *c-type lectin 2-1*, *clec4m* and *cd209*, showed similar magnitudes in both the moribund and survivor fish. A previous study of the immune defense of rainbow trout against *A. salmonicida* suggested that *cd209* is an essential receptor that captures this bacteria [24]. Our results showed a high up-regulation of *cd209* in rainbow trout skin, which is consistent with previous studies in the spleen and head kidney tissues (after 1 and 7 days post infection) [23,24]. The expression of the other CLR (*clec4e*) was significantly higher at 2 dpi compared to 6 dpi (FC = 17.22) and was the highest between all of the CLRs. C-type lectins recognize the carbohydrate patterns on pathogen surfaces, opsonize them or activate complement cascade and may induce signaling cascade, leading to the activation of NF-κB, and thus, inflammatory responses [61,62].

After an infection with *A. salmonicida*, antimicrobial peptides (AMPs) were activated, such as *camp* and hepcidin (*hamp*). *Histone h1* and *camp* showed similar magnitudes of expression in the moribund and survivor fish, which is in agreement with the multi-tissue gene expression analysis after 3 and 13 days of *A. salmonicida* infection in rainbow trout [21]. Otherwise, the expression of *hamp* was higher at 2 dpi and then decreased after six days. The up-regulation of these AMPs was noticed in previous studies; however, in other tissues, *Hamp* was previously up-regulated in the liver of rainbow trout using an ELISA test [63], whereas *histone h1* was detected in Atlantic salmon after *Escherichia coli* infection [64]. In summary, AMPs are pivotal parts of the first line of host defense against pathogens that disrupt pathogenic bacterial membranes and regulate the innate immune response [65].

The success of the immune response depends on the signaling, communication and migration of immune cells. Chemokines and cytokines such as interleukins are small glycoproteins that play crucial roles in inflammation, hematopoiesis and immune cell activation and they induce the migration of leukocytes from blood vessels to inflamed tissues [66,67]. In this study, most of the identified chemokines were differentially expressed only at 2 dpi, and thus included down-regulated *ccl2*, *ccl21* and *cxcl11* and up-regulated *ccl19*, *ccl25*, *ccl28*, *cxcl8* and *ccr1*. However, only *ccl19* showed a statistically significant difference between the groups. In accordance, the expressions of *cxcl8* (*il-8*) and *ccl19* were increased in rainbow trout gill tissue after *A. salmonicida* infection [20]. Previously, studies on the teleost revealed that *ccl19* promotes anti-viral and anti-bacterial defense and inflammation [68]. Further, in this study, we noticed an up-regulation of genes encoding interleukins and their receptors including *il1b*, *il6*, *il11*, *il17d*, *il21*, *il1r2* and *il13ra2*. Contrary to rainbow trout gills, the expression of *il6* was up-regulated in the skin, and according to a previous study, it might be induced by lipopolysaccharides and promote phagocyte proliferation [69]. Further, the expressions of *il1b* and *il11* significantly increased at 2 dpi (FC = 6.49 and FC = 8.14, respectively) and decreased after 6 dpi. *IL1b* is a key pro-inflammatory cytokine, which was also up-regulated at early stages of infection in the gill of rainbow trout [20] and Atlantic cod [27], as well as in the head kidney infected with *A. salmonicida achromogenes* [70]. *IL11* belongs to the *il6* cytokine family and plays a major role in hematopoiesis, and it may show pro- and anti-inflammatory responses in fish [70]. In contrast to other interleukins, *il17d* was repressed in moribund fish and then came back to the control level in the survivors. The decreased expression of *il17d* is similar to that of the early stage of bacterial infection in *Siberia sturgeo* [71]. In summary, these results suggest that the expression of chemokines and interleukins in response to bacterial invasion depends on the type of tissue and post infection time, which has also been suggested in black rockfish (*Sebastes schlegelii*) infected with *A. salmonicida* [72].

At an early stage of bacterial infection, the inflammation process is activated and the inflammatory cells (neutrophils and monocytes/macrophages) secrete cytokines such as *il1b*, *il8*, *il6* and tumor necrosis factor (*tnfα*) into the bloodstream, stimulating the production of acute-phase proteins (APPs) [73]. The APPs are involved in many immune processes such as the inactivation of proteolytic enzymes, the control of the distribution of infectious agents (by eliminating pathogens or by modifying surface targets) and in the recovery of damaged tissues [74]. In this study, several genes encoding APPs, such as *saa1*, serum amyloid a5 (*saa5*), haptoglobin (*hp*), *cbln1*, *lysozyme c* and *lysozyme g*, were significantly elevated after an infection in fish skin. Of these genes, the expression levels of *lysozyme c*, *hamp*, *c3*, *c4* and *steap4* were higher at 2 dpi and decreased after 6 dpi to the control level, whereas the serum amyloids were highly expressed at both 2 and 6 dpi. *SAAs* play roles in inflammation, opsonization, cholesterol transport and the degradation of the extracellular matrix, and they might be useful in monitoring and evaluating health in fish [75]. Previous studies on rainbow trout gill, liver and spleen confirmed the up-regulation of *hp* at 3 dpi [20] and the up-regulation of *saa1* at 3 and 13 dpi [21]. The RT-qPCR analysis from this study also confirmed the up-regulation of *saa1* (Figure 8). Otherwise, the ceruloplasmin level in the plasma decreased in both the moribund and survivor fish, which is in accordance with studies on the Nile tilapia (*Oreochromis niloticus*) after an infection with *A. hydrophila* [73].

Previous studies suggested a correlation of complement cascade to acute phase and inflammatory response [29,76]. This study revealed that in rainbow trout skin at 2 days after infection, the complement and coagulation cascades pathway was activated. The complement components *c3*, *c4*, *c6* and *c7*, the complement c1q-like protein 2 (*c1ql2*) and the complement c5a receptor 1 (*c5ar1*) were up-regulated in the moribund fish, which is in accordance with previous studies on rainbow trout [20,21,23]. Of these, only *c7* was slightly up-regulated in the survivor fish. *C4* plays an important role in classical and lectin pathways, *c1ql2* is the initial protein of the classical complement pathway and *c7* plays an integral role in the formation of the membrane attack complex (MAC) [77]. Further, *c5ar1*

was also raised at 2 dpi, which may promote the development of inflammation through chemotaxis and the degranulation of granulocytes and monocytes [78].

Cell death plays a fundamental role during the homeostasis of the host and the defense against pathogens [79]. Several types of programmed cell death (PCD) such as autophagy, apoptosis, ferroptosis, necroptosis and pyroptosis have been identified and classified in vertebrates [79]. In this study, apoptosis, autophagy and necroptosis processes were enriched in both the moribund and survivor fish. Of these, several genes showed similar expression patterns in both groups, such as bcl2 apoptosis regulator (*bcl2*), caspase 8 (*casp8*), *ctsl1*, gamma-aminobutyric acid receptor-associated protein-like 1 and 2 (*gabarapl1*), rb1 inducible coiled-coil 1 (*rb1cc1*) and *calcoco1*. Members of the Bcl2 family proteins such as antiapoptotic *bcl2* suppress apoptosis, whereas *casp8* is an extrinsic apoptosis initiator and necroptosis suppressor [80]. Other genes were significantly increased at 2 dpi and then decreased to the control level, such as nfkb inhibitor alpha (*nfkbia*), jun proto-oncogene ap-1 transcription factor subunit (*jun*), which is involved in apoptosis, and glutamine synthetase (*glul*), which is involved in necroptosis. Moreover, *tnfα*, which stimulates macrophage activity in fish [81] and activates both necroptosis and apoptosis, was also up-regulated after 2 dpi, and then its expression decreased. Previously, a strong expression of *tnfα* was noticed at 3 days post infection by *A. salmonicida achromogenes* in the head kidney of Artic charr (*Salvelinus alpinus*) [82]. Otherwise, the expressions of other genes involved in apoptosis such as *cyc* and dynein light chain lc8-type 1 (*dynll1*) were similar to the uninfected fish at 2 dpi and then raised at 6 dpi. Apoptosis and necroptosis are vital parts of the host immune defense mechanism, which cleans up damaged cells and plays a role in host–pathogen interactions [83]. Further, the ferroptosis process was enriched in moribund fish. Ferroptosis is a Reactive Oxygen Species (ROS)—a dependent form of inflammatory cell death associated with iron accumulation and lipid peroxidation—that induces an inflammatory immune response in macrophages [84]. Between the genes involved in the regulation of ferroptosis, solute carrier family 3 member 2 (*slc3a2*), spermidine/spermine n1-acetyltransferase 1 (*sat1*), microtubule-associated protein 1 light chain 3 beta (*map1lc3b*), ferritin heavy chain 1 (*fth1*), heme oxygenase 1 (*hmox1*), *alox5ap*, acyl-coa synthetase long chain family member 1 (*acsl1*), *ptgs2* and nfe2 like bzip transcription factor 2 (*nfe2le*) were up-regulated at two days post infection in rainbow trout. In accordance with this, the raised expressions of ferritin and *hmox1* and the induction of ferroptosis in response to *E. coli* infection were studied in grass carp (*Ctenopharyngdon idella*) [85]. Altogether, programmed cell death processes are critical to maintain homeostasis and plays a role in immune response.

The cytoskeleton and extracellular matrix (ECM) play essential roles in cell structure and function. A cytoskeleton is a cellular frame inside a cell, and it plays a role in cell motility and division. The ECM is a complex structural entity surrounding and supporting cells in tissues, and it plays a role in the regulation of intercellular communication, apoptosis, angiogenesis and cell differentiation [86]. Many proteins of the cytoskeleton and ECM interact with each other, and they were mostly decreased in both the moribund and survivor fish. Of these, collagens (*col1a1*, *col1a2*, *col1a3*, *col2a1*, *col5a1*, *col5a2*, *col6a1*, *col11a1 and col12a1*), myosins (*mybph* and *mybpc2*), myomosins (*myom1* and *myom2*) and actins (*actc1* and *actn3*) decreased after *A. salmonicida* infection. Further, serpin family h member 1 (*serpinh1*), a collagen-specific chaperon, was also down-regulated in both groups. However, there were also some genes encoding myosins and myozenin, such as *mylk2*, *myl4*, *myo6* and *myoz2*, that showed up-regulation in 2 dpi and 6 dpi, respectively. Tropomyosin 3 (*tpm3*) was differentially regulated depending on the transcript type in both groups. Moreover, metalloproteinases (*mmp9*, *mmp13* and *mmp19*) were up-regulated in both the 2 dpi and 6 dpi groups. Metalloproteinases are endopeptidases, produced by macrophages, and neutrophils, which are involved in tissue turnover, the degradation of ECM components and inflammatory response [87]. One of the transcript variants of the *mmp9* gene showed a statistically significant difference between the moribund and survivor fish. Previous studies on *mmp9* in fish revealed the role of these metalloproteinase in collagen reorganization

in tissue lesions [88], and its expression was increased in several fish species following *A. hydrophila* infection [89]. Further, differentially expressed genes between the moribund and survivor fish were decorin (*dcn*) and keratins (*krt13* and *krt18*) that were down-regulated at 2 dpi and then increased at 6 dpi.

The results presented in this study add to the growing omics resources investigating the rainbow trout response to *A. salmonicida* infection. The presented differentially expressed genes in the skin might be useful as biomarkers for the molecular diagnosis of furunculosis and in new therapeutic development. However, more studies are still required including next-generation sequencing and the higher resolution of electron microscopy (EM). EM allows for the macroscopic and microscopic lesions in rainbow trout to be visualized and described in order to provide a histological and ultrastructural evaluation of the diseases. Previously, the transmission electron microscopy (TEM) method was used to investigate the skin samples of rainbow trout affected by Red Mark Syndrome (RMS) and provided an overview of the infection progression [90]. A correlation of the molecular and morphological data is necessary to explore *A. salmonicida* infection in the rainbow trout aquaculture.

5. Conclusions

An infection with *A. salmonicida* causes damages in rainbow trout aquaculture. To investigate the transcriptional profile of the skin and adhering skeletal muscle in response to *A. salmonicida* infection in rainbow trout, the 4 × 44 oligonucleotide microarray was applied. We identified differentially expressed genes at 2 and 6 days post infection. Further, we investigated the differences between moribund (2 dpi) and survivor (6 dpi) fish. Our results revealed a divergent expression of many genes involved in the fish immune system, mainly including inflammation, antimicrobial peptides, pattern recognition patterns, acute-phase response proteins, such as serum amyloids, lysozymes, cathelicidin, hepcidin and c-type lectins as well as genes involved in complement cascade after infection. Along with the immune response, we saw the up-regulation of leptin, prostaglandins and eicosanoids related to metabolic reprogramming. Further, a gene expression analysis revealed the differential regulation of genes involved in programmed cell death (apoptosis, necroptosis and ferroptosis) and cytoskeleton and extracellular matrix remodeling. The results from this study confirm the complexity of the response to bacterial infection and constitute a source for further studies on furunculosis in rainbow trout. This is, to our knowledge, the first study that aimed to achieve the gene expression profiling of rainbow trout skin after an *A. salmonicida* challenge.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app132312793/s1, Table S1: List of differentially expressed genes with annotations identified in rainbow trout after infection with *A. salmonicida* in moribund (2 dpi) and survivor (6 dpi) fish; Table S2: List of differentially expressed genes with annotations between moribund (2 dpi) and survivor (6 dpi) fish; Figure S1: GO enrichment analysis of moribund (2 dpi) group. Bubble plot presents top 10 GO subcategories with highest *p*-value in main categories: biological process (BP), cellular component (CC), molecular function (MF). (a) Up-regulated genes. (b) Down-regulated genes; Figure S2: GO enrichment analysis of survivor (6 dpi) group. Bubble plot presents top 10 GO subcategories with highest *p*-value in main categories: biological process (BP), cellular component (CC), molecular function (MF). (a) Up-regulated genes. (b) Down-regulated genes; Figure S3: GO enrichment analysis between moribund (2 dpi) and survivor (6 dpi) groups. Bubble plot presents top 10 GO subcategories with highest *p*-value in main categories: biological process (BP), cellular component (CC), molecular function (MF). (a) Genes decreased over time. (b) Genes raised over time.

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