Transcriptome Analysis of Turbot (*Scophthalmus maximus*) Infected With *Aeromonas salmonicida* Reveals a Direct Effect on Leptin Synthesis as a Neuroendocrine Mediator of Inflammation and Metabolism Regulation

Marta Librán-Pérez, Patricia Pereiro, Antonio Figueras and Beatriz Novoa *

Immunology and Genomics Group, Instituto de Investigaciones Marinas (IIM), Consejo Superior de Investigaciones Científicas (CSIC), Vigo, Spain

Aeromonas salmonicida subsp. *salmonicida* is the causative agent of furunculosis, a disease affecting numerous fish species worldwide. It is a highly pathogenic bacterium for turbot, whose farming production represents an important economic activity in several European countries and China. To better understand the response of this organism to *A. salmonicida*, we conducted RNA-Seq analysis of the head kidney from experimentally infected and uninfected turbot juveniles at 24 hours post-infection (hpi). As expected, among the differentially expressed genes (DEGs) between infected and uninfected fish, we observed the modulation of a multitude of immune-related genes but also a high representation of genes linked to metabolism. Interestingly, one of the most upregulated genes was that encoding the hormone leptin. Leptin is a multifunctional hormone/cytokine that has been shown to play roles in the immune system, stress response, food intake, metabolism and energy balance. We used recombinant human leptin to elucidate its role during infection with *A. salmonicida* in turbot (anorexigenic activity, ability to modulate metabolism and the immune response, and its effect on survival and bacterial load during infection). We found that the intraperitoneal administration of leptin was able to alter the response to the bacteria at the immune level, but especially at the metabolic level, which resulted in a higher survival rate without affecting the bacterial load. Based on this, we hypothesized that leptin could offer great potential as a therapeutic treatment during furunculosis outbreaks by reducing the impact of sepsis. Our results reveal the complex interplay between bacterial activity and the regulation of food intake, metabolism and inflammation.

Keywords: turbot, *Aeromonas salmonicida*, RNA-Seq, leptin, immunometabolism

INTRODUCTION

Bacterial pathogens are a major cause of infectious diseases and mortality in fish and cause important economic losses in aquaculture (Meyer, 1991). The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is the aetiologic agent of classical furunculosis, a disease characterized, among other signs, by sepsis, muscular lesions and haemorrhages and death in fish populations (McCarthy and Roberts, 1980). This pathogen is a serious concern, especially for salmonid species such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Austin and Austin, 2007). However, several studies have shown that *A. salmonicida* can infect many other economically relevant fish species (Nougayrede et al., 1990; Willumsen, 1990; Real et al., 1994; El Morabit et al., 2004; Magariños et al., 2011; Fernández-Álvarez et al., 2016) and even cause serious furunculosis outbreaks in some of them, including turbot (*Scophthalmus maximus*) (Toranzo and Barja, 1992; Pedersen and Larsen, 1996).

Turbot aquaculture is an important economic activity in China and certain European countries, especially Spain (APROMAR, 2020). Epizootic outbreaks of acute furunculosis in turbot farms have been reported in Spain, Denmark, France, Norway and China (Nougayrede et al., 1990; Toranzo and Barja, 1992; Pedersen et al., 1994; Lillehaug et al., 2003; Fan et al., 2005). Because aquaculture is probably the fastest growing food-producing sector in the world, there is an urgent need to find effective treatments against the different pathogens affecting farmed fish. Bacterial outbreaks are usually controlled with antibiotics, which are often neither totally effective nor consumer/environmentally friendly, and there is a potential danger of antibiotic resistance development in aquatic bacteria (Defoirdt et al., 2011; Romero et al., 2012). Therefore, alternative treatments aimed at controlling bacterial diseases are required to guarantee the sustainable development of the aquaculture industry. During the last decade, considerable effort has been devoted to increasing genomic and transcriptomic resources to better understand the immune reactions of fish against bacterial fish pathogens, which is pivotal for developing new antibacterial treatments.

In recent years, a growing interest has emerged in studying the importance of the different metabolic pathways in the regulation of immune responses against pathogens in mammals (O'Neill et al., 2016). Immune cell function and metabolism are closely linked, and alterations in cellular metabolism influence immune cell function; conversely, immune cell function determines the cellular metabolic state (O'Neill et al., 2016). Indeed, currently, it is well known that upon pathogen recognition by immune cell types, cellular metabolic pathways undergo shifts that alter their functionality (O'Neill et al., 2016). On the other hand, viruses and intracellular bacterial pathogens are able to reprogram the metabolism of host cells to favour their own replication (Eisenreich et al., 2019).

However, the effects of metabolism on immune function are not limited to cellular metabolism, but also involve systemic metabolism (Matarese and La Cava, 2004; Wensveen et al., 2019). The neuroendocrine system, metabolism and immune function are intricately linked, with several cytokines, hormones,

neuropeptides and transcription factors performing pivotal functions in food intake, metabolic processes and immune response (Matarese and La Cava, 2004). Among the metabolic hormones, leptin is a well-described adipocyte-derived hormone/cytokine with hormonal and immune actions that link all of those processes (Matarese and La Cava, 2004; Lam and Lu, 2007). The leptin receptor is expressed at high levels in the hypothalamus, but also in endothelial cells, pancreatic β-cells, ovaries and different immune cell types, such as monocytes/macrophages and T and B cells (Matarese and La Cava, 2004). Fat accumulation in adipocytes promotes the synthesis of leptin, which acts as an anorexigenic hormone by signalling satiety to the brain and resulting in the inhibition of food intake (Matarese and La Cava, 2004). At the metabolic level, leptin induces lipolysis, inhibits lipogenesis and systemically increases the metabolism of glucose (Reidy and Weber, 2000). As an immune regulator, leptin promotes the activation of monocytes/macrophages, chemotaxis and activation of neutrophils, development and activation of natural killer cells, T-helper 1 responses and activation and proliferation of B cells, among other immune functions (Matarese and La Cava, 2004; Lam and Lu, 2007).

In fish, duplicated *leptin* (*lep*) genes and a single *leptin receptor* (*lepr*) gene are usually present, with some exceptions (Blanco and Soengas, 2021). Nevertheless, as in mammals, alternative splicing of the *lepr* gene can generate a variety of receptor isoforms with different characteristics (Blanco and Soengas, 2021), which could exert a clear influence on leptin functionality. However, teleost *lep* orthologues are not primarily expressed in adipocytes, with the liver being the main leptin-secreting tissue (Blanco and Soengas, 2021). Numerous publications confirmed a similar role of leptin in teleosts compared to tetrapods: regulation of food intake, metabolism, stress, growth and reproduction (Copeland et al., 2011). However, the effect of this hormone against infection and its effect on metabolism-immune system interplay remain practically unexplored in fish.

By analysing the transcriptome response of head kidney samples of juvenile turbot to *A. salmocida*, we found that, in addition to a multitude of immune genes, one of the genes which was more overexpressed at 24 hpi was *lep*. Therefore, because recombinant human leptin has been previously demonstrated to reproduce some of its effects in fish (Peyon et al., 2001; Weil et al., 2003; de Pedro et al., 2006; Gorissen et al., 2012), we administered it to turbot to explore the biological significance of *lep* overexpression during an infection with bacteria. We found that human leptin was able to reduce food intake, modulate metabolism, and alter the expression of certain metabolism- and immune-related genes in turbot. The activity mediated by recombinant human leptin also significantly increased the survival of the *A. salmonicida*-infected turbot, which highlights the potential of this type of metabolic treatment to ameliorate the outcome of bacterial infections.

MATERIALS AND METHODS

Fish, Bacteria and Recombinant Leptin

Juvenile turbot were obtained from a commercial fish farm (Insuiña S.L., Galicia, Spain). Fish were maintained in 500 L

fibreglass tanks with a recirculating saline water system (total salinity approximately 35 g/L) and a 12 L:12 D photoperiod at 15°C. The fish mean weight was 2.5 g ± 0.3 g. Prior to the experiments, fish were acclimatized to laboratory conditions for 2 weeks. Animals were fed daily to satiety with a commercial dry diet (LARVIVA-BioMar). For fish injections, animals were anaesthetized with MS-222 (50 mg/L); before samplings and at the end of the survival experiments, fish were sacrificed via MS-222 overdose (500 mg/L). Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number ES3605702020012020/13/FUN.01/INM06/BNG.

Aeromonas salmonicida subsp. *salmonicida* (strain VT 45.1, kindly provided by Dr. M.L. Lemos; University of Santiago de Compostela, Spain) was cultured in tryptic soy agar (TSA) plates at 22°C for 24 h before use. A bacterial suspension was prepared in 1x phosphate buffered saline (PBS) immediately before inoculation. Serial dilutions were performed to calculate the number of colony forming units (CFU)/mL.

Recombinant human leptin was purchased from Sigma–Aldrich (L-4146) and dissolved in PBS to the desired concentration. Leptin dose (1 µg/g body weight) and administration procedure (daily intraperitoneal injection) were adapted from previous studies (de Pedro et al., 2006).

Fish Infection and Sampling for RNA–Seq

After two weeks of acclimation, fish were randomly assigned to 60 L experimental tanks. Sedated specimens were intraperitoneally (i.p.) injected with a volume of 50 µL of PBS (control) or with *A. salmonicida* (5.3×10^7 CFU/mL). The head kidneys from 9 fish per condition were sampled at 24 hpi under RNase-free conditions. The same quantity of tissue from 3 animals was pooled, performing 3 biological replicates (3 fish/replicate). Samples were stored at −80°C until RNA isolation.

RNA Isolation and High-Throughput Transcriptome Sequencing

RNA isolation was performed with the Maxwell 16 LEV simplyRNA tissue kit (Promega). The RNA concentration and purity were measured with a spectrophotometer (ND-1000; Nanodrop Technologies Inc., Wilmington, DE, USA), and RNA integrity was analysed in an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. All the samples showed an RNA integrity number (RIN) over 8.0 and were therefore used for Illumina library preparation. Double-stranded cDNA libraries were constructed using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA), and sequencing was performed using Illumina HiSeq 4000 technology at Macrogen Inc., Korea (Seoul, Republic of Korea). The read sequences were deposited in the Sequence Read Archive (SRA) under accession number PRJNA810312.

Bioinformatics Analyses

CLC Genomics Workbench v. 11.0.2 (CLC Bio, Aarhus, Denmark) was used to filter and perform the RNA-Seq and statistical analyses. Raw 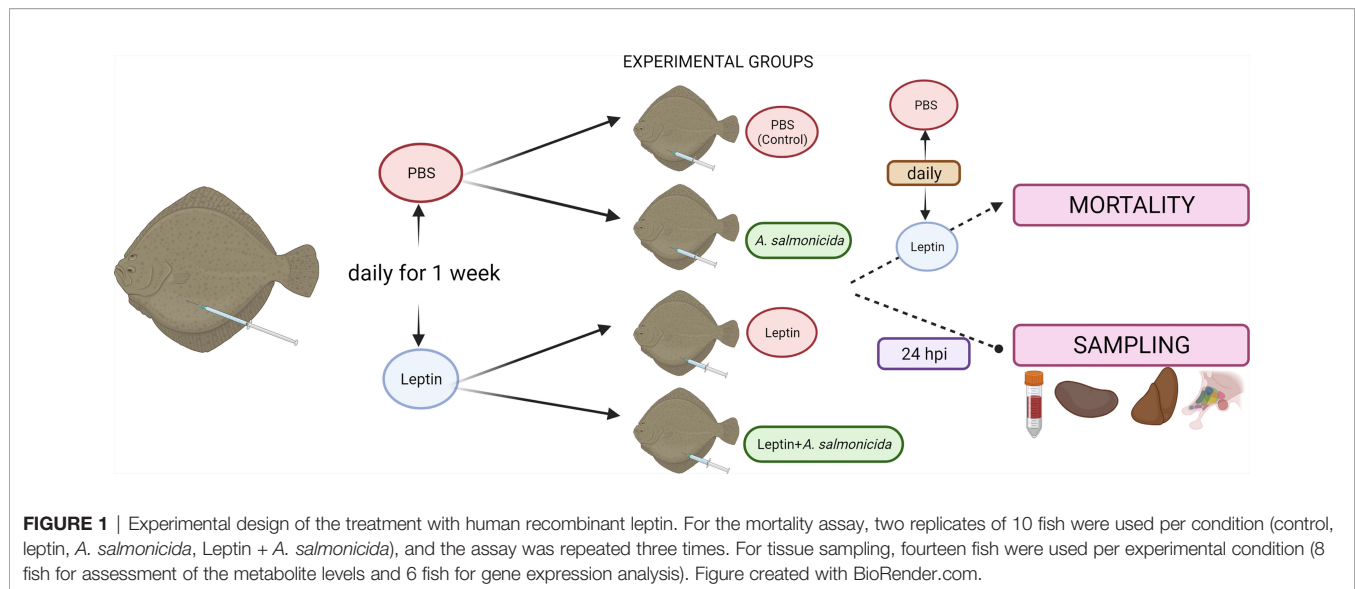reads were trimmed to remove low-quality sequences (quality score limit 0.01 = PHRED 20), and the high-quality reads were used for further analysis. These reads were mapped against the turbot genome (Figueras et al., 2016) with the following parameters: length fraction = 0.8, similarity fraction = 0.8, mismatch cost = 2, insertion cost = 3 and deletion cost = 3. Expression levels were calculated as transcripts per million (TPM) values. To identify and quantify the directions of variability in the data, a principal component analysis (PCA) plot was constructed by using the TPM values for all the genes and with the web tool Clustvis (Metsalu and Vilo, 2015) (https://biit.cs.ut.ee/clustvis/). To determine statistically significant differences between infected and uninfected turbot, a differential expression analysis test was conducted. Genes showing a fold-change>|2| and an FDR<0.05 were selected as differentially expressed genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using OmicsBox software (https://www.biobam.com/omicsbox), and an FDR corrected p value<0.01 was employed. Using the TPM values of the selected DEGs, heatmaps were constructed using the average linkage method with Euclidean distance, also using the web tool Clustvis.

Food Intake Assay

Changes in food intake after bacterial infection and/or leptin administration were evaluated in juvenile turbot. For this, four groups of 10 fish/tank were i.p. inoculated with 50 µL of PBS (controls), leptin (1 µg/g), an *A. salmonicida* suspension (4.3×108 CFU/mL per fish) or leptin + *A. salmonicida*. The experiment was conducted in triplicate. Food intake was registered for 3 days before treatment (to define basal line data) and during the subsequent three days after the administration of the treatments. Food was supplied daily in batches of approximately 5 g every 2 min until satiation. After feeding, the uneaten food remaining at the tank bottom and feed waste were withdrawn, dried and weighed. The amount of food consumed by fish in each tank was calculated as the difference between total food offered and uneaten food. Food intake values registered after treatment are referred to as those of basal values, and the results are shown as the mean ± SEM of the data obtained in three different tanks per treatment.

Survival Assay and Sampling for Metabolite Quantification and Gene Expression Analyses

A schematic representation of the experimental design is shown in **Figure 1**. Forty fish were randomly divided into 2 batches of 20 turbot each. One batch was i.p. injected with 50 µL of PBS, and the other batch was injected with 50 µL of leptin (1 µg/g) every day for one week. After this period, half of the fish from the PBS group (n=10) were i.p. injected with 50 µl of PBS (control group) or an *A. salmonicida* solution (4.3×10^8 CFU/mL) (*A. salmonicida* group). For the leptin-stimulated group, half of the turbot were i.p. injected with leptin (leptin group), and the remaining 10 fish were injected with leptin and *A. salmonicida* (leptin + *A. salmonicida* group). Then, fish were i.p. injected every day with PBS (control and *A. salmonicida* groups) or leptin (leptin and leptin + *A. salmonicida* groups), and mortality was recorded every day. The survival assay was repeated three times, with two replicates per group.

FIGURE 1 | Experimental design of the treatment with human recombinant leptin. For the mortality assay, two replicates of 10 fish were used per condition (control, leptin, *A. salmonicida*, Leptin + *A. salmonicida*), and the assay was repeated three times. For tissue sampling, fourteen fish were used per experimental condition (8 fish for assessment of the metabolite levels and 6 fish for gene expression analysis). Figure created with BioRender.com.

For tissue sampling, a parallel experiment was conducted (**Figure 1**). Groups of 28 turbot were also inoculated daily for a week with leptin or the same volume of PBS. Then, each group was divided into two groups of 14 fish. The fish inoculated with PBS were inoculated again with PBS (control group) or with the bacteria (*A. salmonicida* group). Among the leptin-administered groups, one of them was i.p. injected with leptin (leptin group), and the other one was treated with leptin + *A. salmonicida*. At 24 hpi, fish were anaesthetized with MS-222, and blood was collected by caudal puncture with heparinized syringes. Plasma samples were obtained after blood centrifugation at 400 xg for 10 min, deproteinized immediately (using 0.6 M perchloric acid (PCA)) and neutralized (using 1 M potassium bicarbonate) before their freezing and storage at -80°C until further assay. Fish were sacrificed, and liver, head kidney and hypothalamus samples were taken, snap-frozen in dry ice, and stored at -80°C. Samples from a total of 8 fish were used to assess metabolite levels, whereas the remaining 6 fish were used for gene expression analysis through qPCR.

Assessment of Metabolite Levels

Lactate, glucose and triglyceride levels were determined enzymatically using commercial kits (Spinreact, Barcelona, Spain) adapted to a microplate format. Tissue samples used to assess metabolite levels were homogenized by mechanical disruption using a potter linked to a motor pestle in 7.5 vol of ice-cooled 6% PCA and neutralized (using 1 mol/L potassium bicarbonate). The homogenate was centrifuged at 16,000 xg for 4 min, and the supernatant was collected to determine metabolite levels.

RNA Isolation, cDNA Synthesis and qPCR Analysis

Total RNA was extracted from head kidney, liver and hypothalamus using the Maxwell 16 LEV simplyRNA tissue kit (Promega) with the automated Maxwell 16 instrument in accordance with instructions provided by the manufacturer.

cDNA synthesis was performed with the NZY First-Strand cDNA Synthesis Kit (NZYtech) using 500 ng of RNA. The quantitative analysis of gene expression was determined using the 7300 Real Time PCR System (Applied Biosystems). Specific qPCR primers for turbot genes were designed based on sequences obtained from the turbot genome (Figueras et al., 2016) or transcriptome (Pereiro et al., 2012) by using the Primer3 program (Rozen and Skaletsky, 2000). Their amplification efficiency was calculated using seven serial five-fold dilutions of head kidney cDNA from unstimulated turbot with the threshold cycle (CT) slope method (Pfaffl, 2001). For the detection of *A. salmonicida* subsp. *salmonicida*, we used specific primers designed on the basis of the publication of Balcázar et al. (2007) but including some modifications. Analyses were performed on 1 µL of cDNA using SYBR GREEN PCR Master Mix (Applied Biosystems) in a total PCR volume of 25 µL containing 10 µM of each primer. Sequences of the forward and reverse primers used for each gene expression analysis are shown in **Supplementary Table S1**. Thermal cycling was initiated with a denaturation step (95°C, 10 min) followed by 40 cycles of a denaturation step (95°C, 15 s) and a hybridization-elongation step (60°C, 1 min). The mRNA levels of the target genes were normalized to the reference gene *eukaryotic translation elongation factor 1 alpha* (*eef1a*), which was stably expressed in this experiment. Expression levels were calculated following the Pfaffl method (Pfaffl, 2001).

Statistics

Comparisons among groups were carried out using Student's t test (paired comparisons) or one-way ANOVA (multiple comparisons) followed by a Student-Newman–Keuls test, and differences were considered statistically significant at $p < 0.05$. When necessary, data were log-transformed to fulfil the conditions of the analysis of variance.

In survival experiments, Kaplan–Meier cumulative survival curves were analysed for statistical significance with the log-rank

(Mantel–Cox) test, and significant differences are displayed as *** (0.0001 < p < 0.001), ** (0.001 < p < 0.01) or * (0.01 < p < 0.05).

RESULTS

Transcriptome Modulation in Turbot After *A. salmonicida* Infection

Using the TPM values obtained from RNA-Seq analyses, PCA was performed to determine the sample distribution and to identify the presence of outliers (**Figure 2A**). The PCA plot showed that the control samples were very similar to each other compared with the *A. salmonicida* samples. **Figure 2B** shows the distribution and fold-change magnitude of the differentially expressed genes (DEGs) in the head kidney from infected animals compared to the controls. A total of 2,056 genes were differentially regulated, with 1,142 upregulated and 914 downregulated at 24 hpi (**Figure 2B**; **Supplementary File S1**).

To better elucidate the biological processes in which these DEGs were involved, GO enrichment analyses were carried out. A total of 221 GO terms were significantly enriched according to an FDR<0.01 (**Supplementary File S2**), although only the 40 most statistically significant terms are shown in the graph (**Figure 3A**). As expected, among the most significantly enriched biological processes, a multitude of immune-related terms were represented, including those related to pathogen recognition *via* toll-like receptors (TLRs), macrophage activation, cytokine activities, antigen presentation and inflammation (**Figure 3A**; **Supplementary File S2**). However, several metabolic processes involved in nucleotide, carbohydrate, lipid and amino acid metabolism were also significantly enriched (**Supplementary File S2**). This fact was also manifested in the KEGG pathway analysis, which highlighted the modulation of different metabolic routes after *A. salmonicida* challenge (**Figure 3B**). Among these, amino acid, lipid, nucleotide and carbohydrate metabolism were the most enriched KEGG pathways.
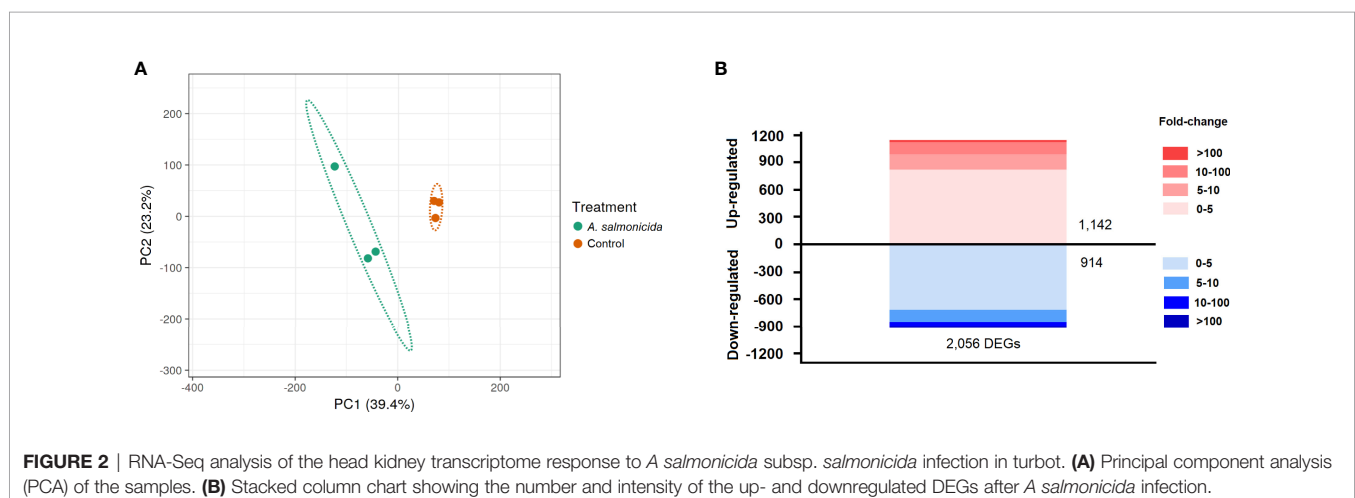
Since glucose and lipid metabolism are intimately linked and have been described as the major metabolic processes impacted upon host responses to infection (Gleeson and Sheedy, 2016), heatmaps were constructed to show the alteration in the gene expression profiles of both metabolic processes after *A. salmonicida* infection. When those DEGs related to carbohydrate metabolism were represented, we observed that, in general terms, glycogenolysis and glycolysis seemed to be inhibited at 24 hpi (**Figure 4A**). Nevertheless, the existence of different isoforms for some glycolytic enzymes, such as hexokinases and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatases, with alternative functions, reflects an opposite regulation in head kidney. On the other hand, when hormones and hormone receptors playing major roles in glucose and lipid metabolism were analysed, downregulation of both insulin and growth hormone receptors (*insr* and *ghr*) was observed after bacterial challenge, whereas the *leptin* (*lep*) gene was highly induced by *A. salmonicida* (**Figure 4B**). Finally, certain genes involved in lipid metabolic processes were also affected by challenge with the bacteria (**Figure 4C**).

Among the annotated DEGs more substantially modulated in head kidney after *A. salmonicida* infection (**Table 1**), a significant number of immune genes were represented among the upregulated DEGs. Interestingly, the fifth most upregulated gene was the *lep* gene, with an FC=270.89. Among the top 25 downregulated and annotated DEGs, an important representation of cytoskeleton organization proteins was observed, including different myosin and troponin members (**Table 1**).

Protective Effect of Leptin During Infection With *A. salmonicida* and Modulation of the Immune Response

To better elucidate the potential role of leptin during infection with *A. salmonicida*, we evaluated whether recombinant human leptin showed any protective or pernicious effect during a challenge with the bacteria. After infection, those fish inoculated with *A. salmonicida* alone showed a survival rate of 20.78%, whereas those turbot infected but treated with leptin achieved a survival rate of 54.17% (**Figure 5A**). No mortality events were registered for the uninfected groups. Interestingly,

FIGURE 2 | RNA-Seq analysis of the head kidney transcriptome response to *A salmonicida* subsp. *salmonicida* infection in turbot. **(A)** Principal component analysis (PCA) of the samples. **(B)** Stacked column chart showing the number and intensity of the up- and downregulated DEGs after *A salmonicida* infection.
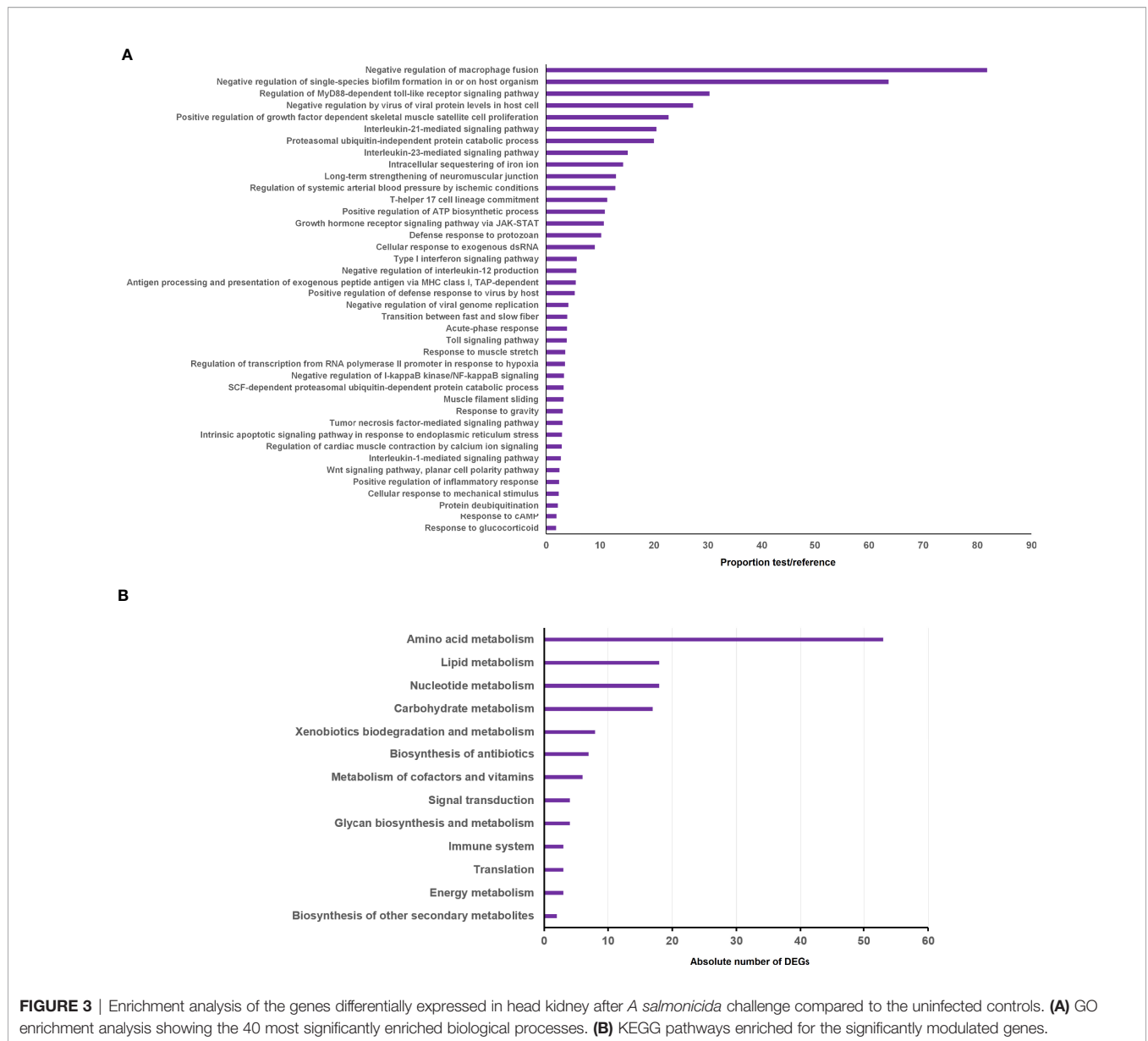
FIGURE 3 | Enrichment analysis of the genes differentially expressed in head kidney after *A salmonicida* challenge compared to the uninfected controls. **(A)** GO enrichment analysis showing the 40 most significantly enriched biological processes. **(B)** KEGG pathways enriched for the significantly modulated genes.
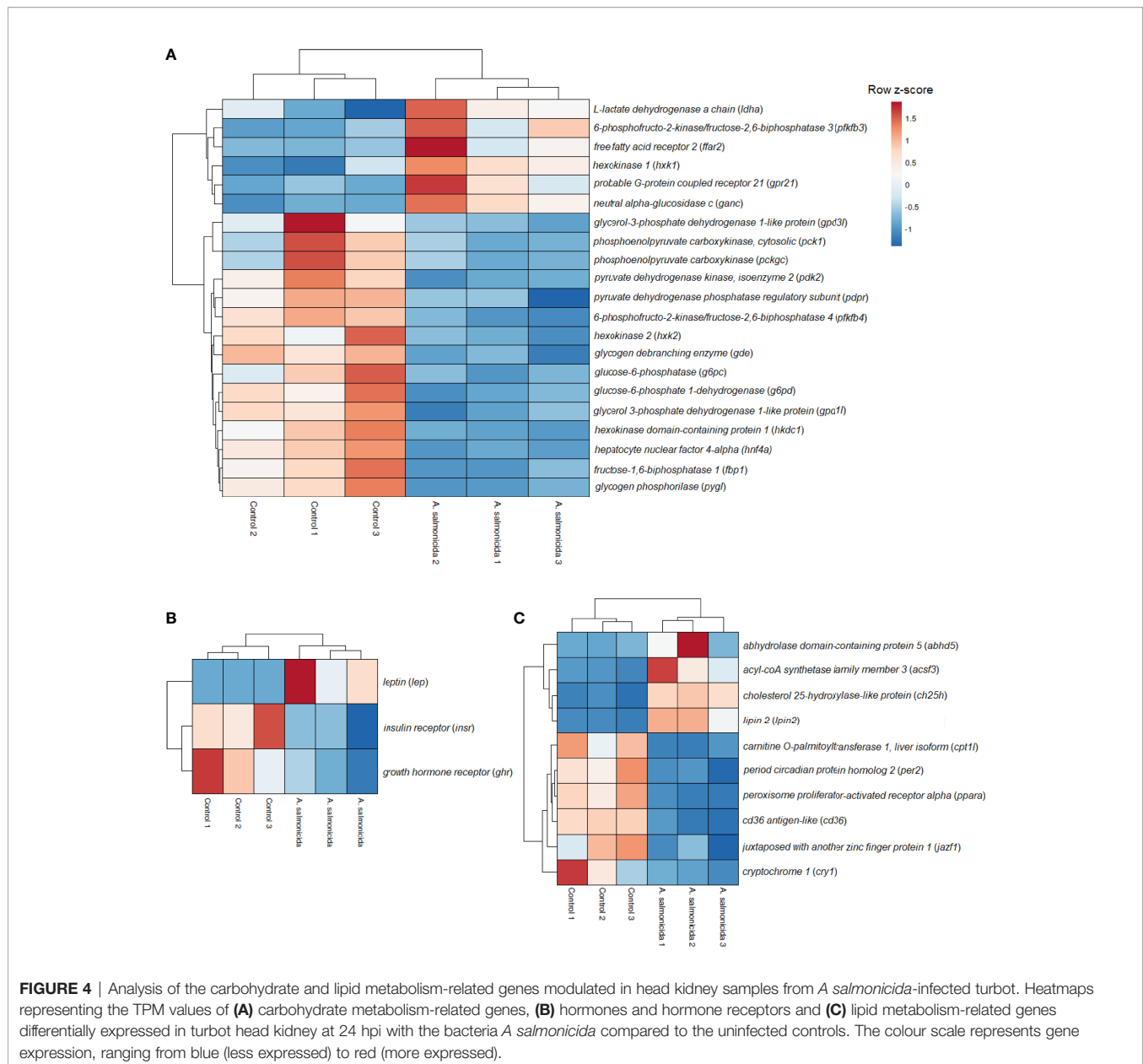
the quantification of the bacteria through qPCR in head kidney samples at 24 hpi revealed that there were no significant differences in the specific detection of *A. salmonicida* subsp. *salmonicida* between those fish receiving leptin and the bacteria and those inoculated with the bacteria alone (**Figure 5B**). Indeed, the results showed a tendency towards higher detection in those animals treated with leptin.

To determine whether the continuous administration of human leptin had the ability to modulate the transcription of the turbot *lep* gene and the immune response in head kidney by itself or during an infection with *A. salmonicida*, we conducted qPCR analyses of selected inflammation-related genes (**Figure 5C**). The exogenous administration of human leptin did not affect the expression of the turbot *lep* gene, which was only induced as a consequence of bacterial infection.

Whereas the challenge with *A. salmonicida* significantly induced the expression of *ifng*, *il1b* and *il4*, leptin treatment significantly reduced the expression of *il6* and *tnfa* compared with the untreated controls (**Figure 5C**). Interestingly, recombinant leptin also significantly reduced the transcription level of *tnfa* in those animals receiving leptin + *A. salmonicida* treatment compared to those inoculated with the bacteria alone (**Figure 5C**).

Recombinant Human Leptin Shows Anorexigenic Effects in Turbot

We also sought to elucidate whether recombinant human leptin is able to affect food intake control in turbot. For this, food intake, gene expression of some anorexigenic and orexigenic neuropeptides and glucose and lactate levels in turbot

FIGURE 4 | Analysis of the carbohydrate and lipid metabolism-related genes modulated in head kidney samples from *A salmonicida*-infected turbot. Heatmaps representing the TPM values of **(A)** carbohydrate metabolism-related genes, **(B)** hormones and hormone receptors and **(C)** lipid metabolism-related genes differentially expressed in turbot head kidney at 24 hpi with the bacteria *A salmonicida* compared to the uninfected controls. The colour scale represents gene expression, ranging from blue (less expressed) to red (more expressed).

hypothalamus were determined (**Figure 6**). Leptin significantly decreased food intake after 1, 3 and 5 days postinfection (dpi) compared with the untreated controls, whereas infection with *A. salmonicida* significantly reduced food intake after 3 and 5 dpi (**Figure 6A**). Furthermore, the decrease was greater in the group treated with leptin and infected with *A. salmonicida* at the three sampling points, reflecting a synergic effect (**Figure 6A**).

Since it is known that leptin inhibits the expression of orexigenic neuropeptides, such as the *neuropeptide y* gene (*npy*) but induces the expression of anorexigenic genes, such as the *proopiomelanocortin* gene (*pomc*) at hypothalamic level, we analyzed the modulation of these two genes after *A. salmonicida* infection and/or leptin administration. In the hypothalamus, treatment with leptin in infected fish significantly decreased

the mRNA levels of the *npy* gene compared with those in controls and infected fish (**Figure 6B**). In contrast, leptin and bacteria alone or in combination were able to significantly increase the mRNA levels of the *pomc* gene (**Figure 6B**). The glucose levels in the hypothalamus increased after treatment with leptin when compared with those in the other groups, and lactate levels increased after treatment with leptin and leptin + *A. salmonicida* (**Figure 6C**).

Leptin and *A. salmonicida* Induce Changes in the Levels of Metabolites and in the Expression of Glucose Regulator Genes

Since a multitude of metabolism-related genes were found to be altered in the RNA-Seq analyses conducted to compare

TABLE 1 | Top 25 up- and down-regulated DEGs in the head kidney at 24 hpi.

Up-regulated (*A. salmonicida*-infected vs. control)		Down-regulated (*A. salmonicida*-infected vs. control)	
Gene ID	**FC**	**Gene ID**	**FC**
Endonuclease domain-containing 1 protein (ENDD1_HUMAN)	931.67	Myosin-2 (MYH2_HORSE)	-1521.61
Pepsin A (PEPA_SUNMU)	486.61	Myosin heavy chain, fast skeletal muscle (MYSS_CYPCA)	-1266.72
Importin subunit alpha-5 (IMA5_XENLA)	413.60	Myosin-13 (MYH13_HUMAN)	-569.44
Interleukin-1 receptor type 2 (IL1R2_CHLAE)	302.31	Low choriolytic enzyme (LCE_ORYLA)	-175.01
Leptin (LEP_TETNG)	270.89	Myosin regulatory light chain 2, skeletal muscle isoform (MLRS_CHICK)	-65.04
C-X-C motif chemokine 10 (CXL10_BOVIN)	260.55	Troponin I, cardiac muscle (TNNI3_HORSE)	-47.02
Interleukin-10 (IL10_CHICK)	201.43	Parvalbumin beta (PRVB_SCOSC)	-44.09
Toll-like receptor 5 (TLR5_HUMAN)	193.99	Myosin-7 (MYH7_PAPHA)	-39.69
Tumor necrosis factor receptor superfamily member 6B (TNF6B_HUMAN)	184.13	Hyaluronan and proteoglycan link protein 4 (HPLN4_MOUSE)	-38.59
Plasminogen activator inhibitor 1 (PAI1_PIG)	157.03	Inactive phospholipase D5 (PLD5_MOUSE)	-32.76
Haptoglobin (HPT_CAPIB)	136.13	Laminin subunit alpha-3 (LAMA3_MOUSE)	-27.45
L-amino-acid oxidase (OXLA_SIGCA)	132.16	Cartilage matrix protein (MATN1_CHICK)	-24.92
C-C motif chemokine 4 (CCL4_RAT)	131.33	Xin actin-binding repeat-containing protein 2 (XIRP2_RAT)	-24.60
Mitotic interactor and substrate of (PLK1 MISP_HUMAN)	102.26	Troponin I, fast skeletal muscle (TNNI2_CHICK)	-23.40
Probable alpha-ketoglutarate-dependent hypophosphite dioxygenase (HTXA_PSEST)	99.95	Solute carrier family 41 member 2v (S41A2_MACFA)	-21.46
Hepcidin-1 (HEPC1_SALSA)	80.01	Carbonic anhydrase 4 (CAH4_RAT)	-20.44
Cis-aconitate decarboxylase (IRG1_HUMAN)	68.56	CMRF35-like molecule 1 (CLM1_HUMAN)	-19.56
Leukocyte cell-derived chemotaxin-2 (LECT2_MOUSE)	63.44	Sodium- and chloride-dependent transporter XTRP3A (S620A_MOUSE)	-19.49
Neoverrucotoxin subunit alpha (STXA_SYNVE)	57.49	Pentraxin fusion protein (PXN1_XENLA)	-18.14
Suppressor of cytokine signaling 1 (SOCS1_MOUSE)	56.20	Down syndrome cell adhesion molecule-like protein 1 (DSCL1_HUMAN)	-18.12
Programmed cell death 1 ligand 1 (PD1L1_HUMAN)	55.47	Aquaporin-4 (AQP4_MOUSE)	-17.25
Interleukin-1 beta (IL1B_ONCMY)	54.68	Sodium- and chloride-dependent neutral and basic amino acid transporter B(0+) (S6A14_HUMAN)	-17.23
Tumor necrosis factor receptor superfamily member 6B (TNF6B_HUMAN)	52.87	Myosin-binding protein H (MYBPH_CHICK)	-15.65
Interleukin-27 subunit beta (IL27B_HUMAN)	49.23	Alpha-tectorin (TECTA_MOUSE)	-15.62
Cell adhesion molecule 3 (CADM3_XENLA)	47.64	Actin-binding Rho-activating protein (ABRA_PIG)	-15.27

A. salmonicida-infected and uninfected turbot, and due to the involvement of leptin in metabolic regulation, we evaluated how both treatments or their combination affected different metabolic parameters (**Figure 7**). The measurement of the levels of metabolites (glucose, lactate and triglycerides) in plasma, head kidney and liver revealed interesting modulations of energy expenditure (**Figure 7A**). Those turbot treated with leptin showed that glucose levels significantly increased in plasma and head kidney, whereas the glucose level was reduced in liver; lactate was only affected in plasma, with a significant increase in those animals receiving leptin (**Figure 7A**). In contrast, triglyceride levels were reduced in plasma, head kidney and liver by leptin administration (**Figure 7A**). Interestingly, *A. salmonicida* challenge reduced the glucose levels in plasma and liver, but the decrease in the plasma glucose levels after infection was counteracted in the presence of leptin, which elevated the levels of plasma glucose even beyond the level of the absolute control (**Figure 7A**). On the other hand, lactate and triglyceride levels were not significantly affected by the bacterial challenge. In general terms, the presence of leptin during the infection tended to modulate the metabolite levels towards those observed in the presence of leptin alone (**Figure 7A**). Leptin treatment (in the presence or absence of infection) induced the upregulation of the *glucose transporter 2* (*glut2*) gene in the liver, especially with the combination of both treatments (**Figure 7B**). On the other hand, the expression of the glycolysis initiating enzyme *glucokinase* (*gck*)

gene in the liver was significantly reduced by the bacteria, but especially by the administration of leptin (**Figure 7B**). The *fatty acid synthase* (*fasn*) gene was inhibited both by leptin administration and bacteria, whereas *carnitine palmitoyltransferase 1a* (*cpt1a*) was inhibited by the bacteria alone but induced by leptin administration (in the presence or absence of infection) (**Figure 7B**).

DISCUSSION

High-throughput molecular analyses (genomics, transcriptomics, proteomics or metabolomics) allow us to obtain a diversity of information that can be used to further develop potential preventive strategies or treatments against infectious diseases. For that reason, we wanted to study the overall transcriptome response of turbot to infection with the bacterium *A. salmonicida*, one of the most threatening pathogens in the culture of this important commercial fish species.

Some studies have used microarrays or high-throughput RNA sequencing (RNA-Seq) to understand the effects of *A. salmonicida* subsp. *salmonicida* infection in fish, including rainbow trout (Rebl et al., 2014; Long et al., 2015; Ji et al., 2020), Atlantic salmon (Ewart et al., 2005; Xia et al., 2022) and turbot (Millán et al., 2011). Millán et al. (2011), using an immune-enriched oligo-microarray, partially characterized the
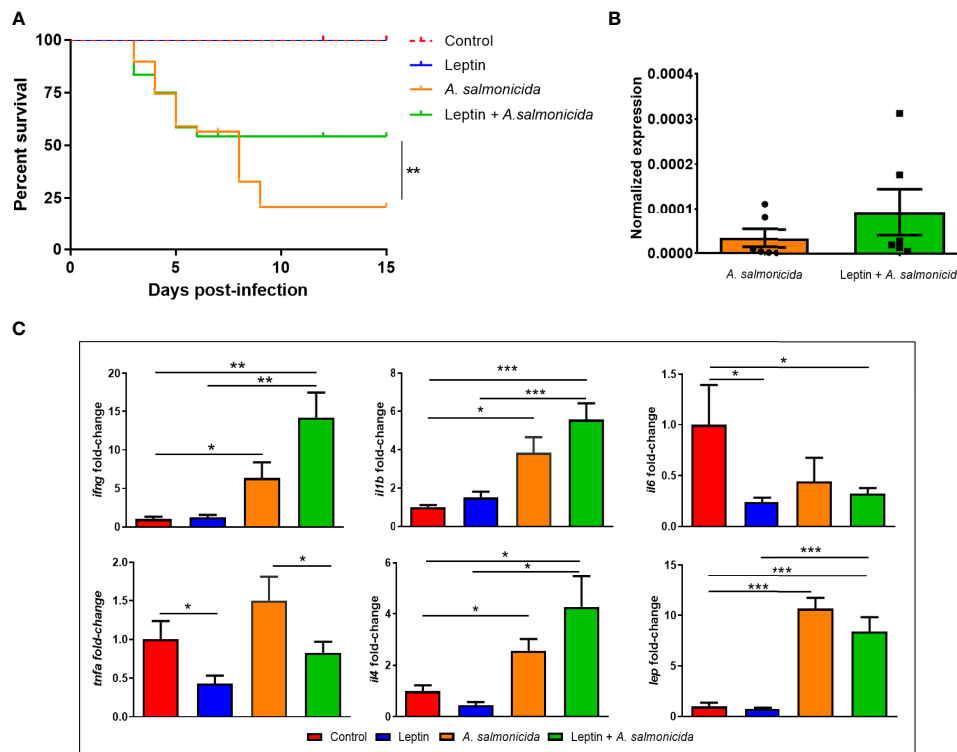
FIGURE 5 | Effect of the chronic administration of human recombinant leptin on the survival, bacterial load and expression of inflammation-related genes in head kidney during an infection with *A salmonicida* in juvenile turbot. **(A)** Kaplan–Meier survival curves of fish infected with the bacteria and treated or not treated with leptin and the corresponding uninfected controls. The graph represents the results of one of the three independent experiments conducted (n = 20). Survival curves were analysed for statistical significance with the log-rank (Mantel–Cox) test. Statistically significant differences are displayed as ** (0.01≤ p ≥ 0.001). **(B)** qPCR detection of *A salmonicida* subsp. *salmonicida* in head kidney samples from infected fish at 24 hpi. The bacterial signal was normalized to the expression of *eef1a*. **(C)** Gene expression analysis of inflammation-related cytokines in head kidney at 24 hpi. The expression level of each gene was normalized to the expression of *eef1a* and expressed as the fold-change with respect to the levels detected in the control group. Data represent the mean + SEM of 6 individuals. Statistically significant differences are displayed as *(p ≤ 0.05), **(p ≤ 0.01) or ***(p ≤ 0.001).

transcriptome response of different tissues (spleen, liver and head kidney) from turbot to infection with *A. salmonicida*. However, that microarray was biased towards certain immune-related genes, and the overall response remained unexplored. Therefore, the present study is the first to conduct in-depth transcriptome analysis in turbot after infection with *A. salmonicida*.

As expected, RNA-Seq analysis of head kidney samples from *A. salmonicida*-infected and uninfected turbot revealed a multitude of immune DEGs upregulated during the infection (interleukins, chemokines, toll-like receptors, antigen-presentation proteins, complement components, etc.), as well as numerous enriched biological processes related to different aspects of the immune response. These modulations were compatible with the activation of a wide range of antibacterial mechanisms (Magnadóttir, 2006). However, the most downregulated genes were mainly involved with the cytoskeleton (myosins, actins, troponins) and extracellular matrix organization. This fact is frequently observed during infection with pathogenic bacteria, which use bacterial virulence factors to hijack the host cell machinery and

manipulate cytoskeleton and tight junction integrity to facilitate their attachment, entry into cells, movement within and between cells, and avoidance of phagocytosis, among other processes (Navarro-García et al., 2013).

Interestingly, a multitude of metabolism-related genes were also differentially modulated after infection, reflecting the tight link between metabolism and immunity. The metabolic gene showing the greatest modulation was the *leptin (lep)* gene, which was overexpressed 271-fold in *A. salmonicida*-infected turbot compared to the controls. Leptin is a pleiotropic hormone involved in the regulation of food intake and body fat through its effects on the central nervous system (CNS) *via* neuroendocrine signalling systems (Francisco et al., 2018). However, leptin also exerts powerful peripheral modulations on immune cells (Francisco et al., 2018; Maurya et al., 2018). Consequently, it is considered to participate in the interplay between the immune response and metabolism (Lam and Lu, 2007; Francisco et al., 2018). In humans, this hormone is secreted mainly by white adipose tissue, while the main tissue involved in leptin synthesis in teleost fish is the liver, and its levels are positively correlated with the amount of body fat (Considine
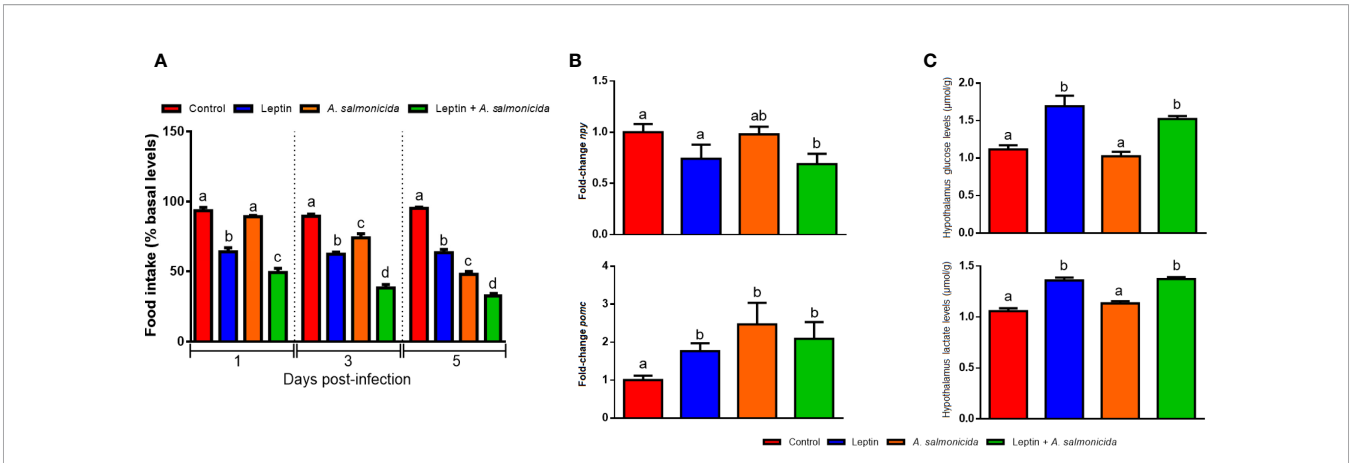
FIGURE 6 | Anorexigenic effects of recombinant human leptin. **(A)** Effect of the chronic administration of leptin on food intake in the absence or presence of *A salmonicida* infection. Food intake is represented as the percentage of food ingested with respect to basal levels (calculated as the average of food intake during the three days prior to the experiment). The results are shown as the mean + SEM of the results obtained in three different experiments in which 10 fish were used per group in each experiment. Different letters indicate significant differences (p < 0.05) among treatments at the same post-treatment time. **(B)** Gene expression analysis of *npy* and *pmoc* and **(C)** glucose and lactate levels in the hypothalamus at 24 h after the i.p. administration of saline solution (control), leptin, *A salmonicida* or leptin + *A salmonicida*. For the gene expression data, the expression level of each gene was normalized to the expression of *eef1a* and expressed as the fold-change with respect to the levels detected in the control group. Each value represents the mean + SEM of n = 6 fish per treatment. Different letters indicate significant differences (p < 0.05) among treatments.

et al., 1996; Blanco and Soengas, 2021). Mammalian immune cells express leptin receptors, and therefore, leptin is able to exert a variety of immune functions, such as activation of monocytes/ macrophages and dendritic cells, enhancement of NK-cell cytotoxicity, cytokine induction, chemotaxis, shift of T cells towards the Th1 phenotype and proliferation of naïve T cells (Francisco et al., 2018). Most of these immune effects are mediated by the induction of certain proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 or IFN-γ, and the

inhibition of anti-inflammatory cytokines, such as IL-4 (Francisco et al., 2018; Maurya et al., 2018).

In murine models, leptin deficiency has been associated with increased susceptibility to bacterial infections and mortality, whereas leptin supplementation had the opposite effect (Ikejima et al., 2005; Wieland et al., 2005; Hsu et al., 2007; Tschöp et al., 2010). In this work, we sought to determine whether *lep* overexpression after *A. salmonicida* infection in turbot could be related to infection prognosis. Although teleost
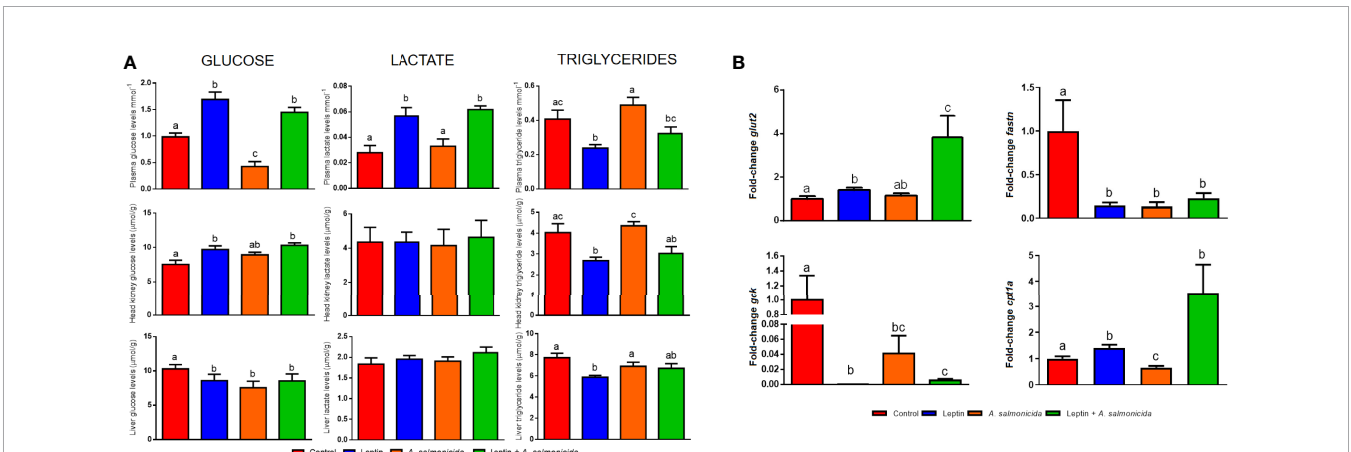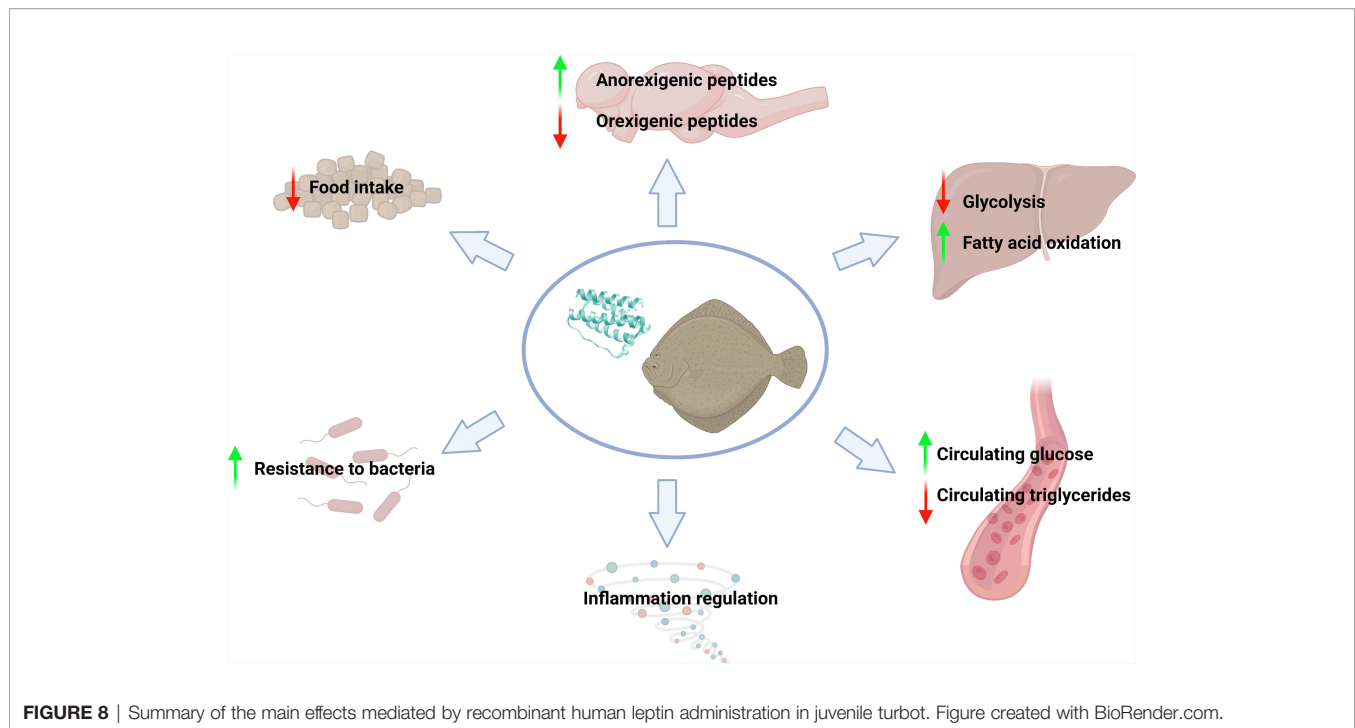

FIGURE 7 | Measurement of metabolic regulation induced by leptin and *A salmonicida*. **(A)** Levels of glucose, lactate and triglycerides in plasma, head kidney and liver, and **(B)** gene expression analysis of *glut2*, *gck*, *fasn* and *cpt1a* in liver of turbot chronically treated with leptin in the absence or presence of infection. Fish were sampled at 24 hpi, where 8 individuals were used for assessment of the metabolite levels and 6 fish were used for gene expression analysis. For the gene expression data, the expression level of each gene was normalized to the expression of *eef1a* and expressed as the fold-change with respect to the levels detected in the control group. Graphs represent the mean + SEM of 8 or 6 fish per treatment, respectively. Different letters indicate significant differences (p < 0.05) among treatments.

FIGURE 8 | Summary of the main effects mediated by recombinant human leptin administration in juvenile turbot. Figure created with BioRender.com.

and mammalian leptins show very low sequence identity, their tertiary structures and key amino acids required for their biological activity are highly conserved (Blanco and Soengas, 2021). Because of this, we used recombinant human leptin to study its effect in turbot and its contribution to the bacterial infection outcome. We found that the chronic administration of recombinant human leptin for one week before bacterial challenge and during the course of bacterial infection significantly increased survival after *A. salmonicida* infection. Curiously, the detection of *A. salmonicida* in head kidney samples did not show significant differences between those animals receiving leptin and those inoculated with PBS, reflecting that the protective mechanisms induced by leptin could not be related to a boosted immune response. Those turbot infected with the bacteria showed a substantial increase in the expression of the proinflammatory cytokines *ifng*, *il1b* and *tnfa*. However, contrary to what was expected due to the proinflammatory effects described for leptin, this hormone downregulated the expression of *il6* and *tnfa* in the absence or presence of infection, which could indicate an anti-inflammatory role of leptin. Moreover, leptin was able to counteract the overexpression of *tnfa* induced by the bacteria. Because of this, the protective effect mediated by leptin seems not to be linked, or is at least not restricted, to the activation of the immune response and could induce a protective effect against sepsis-induced mortality. This protective effect of leptin against septic shock has been previously documented in murine models (2000; Faggioni et al., 1999; Madiehe et al., 2003; Wang et al., 2004; Tschöp et al., 2010; Dong et al., 2013; Landgraf et al., 2014; Vallejos et al., 2018). Indeed, TNF-α administration induces anorexia (among other mechanisms through the activation of

leptin synthesis), metabolic alteration, inflammation and even septic shock and death in mice (Tracey, 1992; Takahashi et al., 1999), which could be consistent with the transcriptome data observed in turbot after *A. salmonicida* infection. Overproduction of TNF-α during septic shock triggers tissue injury, whereas lower levels of this cytokine are related to effective tissue homeostasis and wound healing (Tracey, 1992). More interestingly, anti-TNF-α therapy for patients with sepsis was demonstrated to improve survival (Lv et al., 2014). Since it has been described that leptin mediates protective effects against the toxicity exerted by TNF-α (Takahashi et al., 1999), our results could reflect that the downregulation of the *tnfa* gene in those animals receiving exogenous leptin could be one of the protective effects induced by leptin during infection with *A. salmonicida*. However, we cannot discard a certain effect on the immune response as a consequence of the administration of a non-self protein. Nevertheless, we would expect an increase in the inflammatory response as a consequence of this.

Anorexia is a highly conserved central process of sickness behaviour during infection regulated by immune–endocrine interactions (Wensveen et al., 2019). Fasting has been proposed to be pivotal for host defence against bacterial pathogens, while nutritional supplementation is detrimental in bacterial sepsis (Wang et al., 2016; Wensveen et al., 2019). As expected, *A. salmonicida* infection reduced food intake in turbot, and this effect was potentiated by the administration of human leptin. However, in addition to metabolic alterations as a consequence of reduced food intake, leptin also regulates carbohydrate and lipid metabolism centrally and peripherally (Reidy and Weber, 2000). Evidence indicates that proinflammatory cytokines released upon infection, such as

TNF-α, IL-1β or IL-6, contribute to the development of alterations in glucose and lipid metabolism homeostasis (Shi et al., 2019; Wensveen et al., 2019). Bacterial sepsis affects the ability of host tissues to use glucose *via* glycolysis and alternative fuel sources, such as ketone bodies and free fatty acids, *via* oxidative phosphorylation (Agwunobi et al., 2000). These metabolic alterations, frequently observed during sepsis, represent a prognostic marker for sepsis severity (Wasyluk and Zwolak, 2021). Leptin switches the fuel source from carbohydrate (glucose) to lipid (fatty acids), serving as a powerful inductor of lipolysis and fatty acid oxidation (Reidy and Weber, 2000), and this shift has been shown to be protective during bacterial sepsis (Wang et al., 2016).

We found that *A. salmonicida* infection impacted the expression of a multitude of metabolism-related genes and significantly diminished the liver and plasma levels of glucose. Leptin administration restored and even increased the plasma circulating levels of glucose with respect to the control turbot. Leptin also increased the glucose and lactate levels in the hypothalamus, and glucose in the head kidney, but lower levels of this metabolite were observed in the liver. Additionally, the expression of the gene *gck*, encoding the enzyme catalysing the first reaction of the glycolytic pathway, was inhibited by leptin, and the gene *glut2*, encoding a bidirectional glucose transporter across hepatocytes, was induced by leptin treatment. These expression results could be compatible with glycolysis inhibition by leptin and the release of glucose from the liver to the circulation. On the other hand, although plasma, head kidney and liver triglyceride levels were not affected by the bacteria, leptin decreased their levels, probably due to its role as an inducer of fatty acid oxidation, which is also consistent with the downregulation of *fasn* and the upregulation of *cpt1a*, which are involved in fatty acid synthesis and fatty acid oxidation, respectively.

The higher plasma glucose levels observed due to leptin administration could therefore be a consequence of the shift from glycolysis to fatty acid oxidation, reducing glucose utilization. Indeed, treatment with the glucose analogue 2-deoxy-D-glucose (2DG) improves the survival of mice suffering LPS-induced endotoxaemia or infected with the bacterium Listeria monocytogenes by inducing tissue tolerance to inflammatory damage and consequently reducing organ failure (Wang et al., 2016). On the other hand, a glucose surplus promoted tissue damage in endotoxaemia (Wang et al., 2016). However, higher plasma triglyceride levels were also associated with a protective effect in host tissues during sepsis (Luan et al., 2019). Most likely due to the higher fatty acid oxidation and anorexia induced by leptin, triglyceride levels were lower in plasma, head kidney and liver from fish treated with this hormone. Nevertheless, this reduction in triglyceride levels could result in a benefit for the host in the context of leptin treatment, since triglycerides induce leptin resistance by inhibiting leptin transport across the blood–brain barrier (Banks et al., 2004). Therefore, as was previously observed for the anorexigenic cytokine growth differentiation factor-15 (GDF15) both in mammals (Luan et al., 2019) and fish

(Pereiro et al., 2020), leptin could also induce protective effects after bacterial infection without affecting the bacterial load by acting as a sepsis tolerance mediator that protects host tissues from exacerbated inflammation through the regulation of metabolic fluxes. The metabolic regulation mediated by leptin could also be involved in the inhibition of *tnfa* gene expression, since TNF-α is also a potent regulator of lipid metabolism (Chen et al., 2009). Interestingly, overexpression of gdf15 also reduced the expression of *tnfa* in zebrafish (Pereiro et al., 2020). Since TNF-α increases fatty acid production and, consequently, plasma triglycerides, the lower levels of triglycerides observed in turbot inoculated with recombinant leptin could also be a consequence of *tnfa* downregulation.

CONCLUSIONS

This is the first time that the overall transcriptomic response of turbot infected with *A. salmonicida* has been described. In addition to the expected immune response induced by the bacteria, numerous metabolic genes were found to be altered by the infection. Among these, the gene encoding the hormone leptin showed strong upregulation, which highlights the relevance of metabolic regulation during infection. Treatment with recombinant human leptin before and during the course of infection with *A. salmonicida* significantly increased the survival of the treated turbot, but without affecting the bacterial load. Recombinant leptin significantly reduced food intake and potentiated anorexia in the infected turbot. Moreover, exogenous leptin restored the plasma glucose level reduced by the bacteria and even elevated it over that observed in the control fish, which could indicate leptin mediation of glycolysis inhibition. On the other hand, leptin inhibited the level of circulating triglycerides, which could be linked to the inhibition of *tnfa* gene expression, among other modulatory effects. The main effects mediated by leptin in turbot and described in this work were summarized in **Figure 8**. Based on these results, it seems that leptin protected turbot from *A. salmonicida* infection through the regulation of the interplay among immunity, food intake and systemic metabolism, probably resulting in an increase in sepsis tolerance. More investigations are needed to better elucidate the complete repertoire of mechanisms mediated by leptin in the context of bacterial infection. However, leptin administration could be suggested as an interesting therapeutic treatment for bacterial disease outbreaks in aquaculture.

DATA AVAILABILITY STATEMENT

ETHICS STATEMENT

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.888115/full#supplementary-material

Supplementary File S2 | Biological process GO terms enriched for the comparison *A. salmonicida*-infected vs. control turbot (FDR<0.01).

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