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RECEIVED 06 July 2025
ACCEPTED 22 October 2025
PUBLISHED 17 November 2025

CITATION
Levipan HA, Wicki H, Barrios-Henríquez F,
Pozo-Solar F, Irgang R and
Avendaño-Herrera R (2025) Gene expression
patterns of immune markers in rainbow trout
during the early stages of seawater infections
with *Piscirickettsia salmonis*.
Front. Vet. Sci. 12:1660383.
doi: 10.3389/fvets.2025.1660383

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Gene expression patterns of immune markers in rainbow trout during the early stages of seawater infections with *Piscirickettsia salmonis*

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Piscirickettsiosis is a fish disease caused by the facultative intracellular bacterium *Piscirickettsia salmonis*. This research aimed to study the immune response of rainbow trout (*Oncorhynchus mykiss*) during the first seawater farming stage upon infection with *P. salmonis*. Fish were challenged by immersion with *P. salmonis* type strain LF-89^T (genogroup 1) and the field isolates Psal-103 (LF-89-like genotype or genogroup 3) and Psal-104 (EM-90-like genotype or genogroup 4). A group of fish was treated with sterile AUSTRAL-SRS (Salmon Rickettsial Septicemia) medium. This group and fish from the infectious treatment and negative control were kept in a pilot-scale recirculating aquaculture system (RAS) for monitoring purposes. The *P. salmonis* load in trout skin and seawater was measured by reverse transcription-quantitative PCR (RT-qPCR) targeting an internal transcribed spacer (ITS) region. ITS transcripts were undetectable in trout skin samples before challenges and in trout skin from the sterile SRS medium treatment after challenges. The number of ITS transcripts in RAS seawater samples was $2.21 \times 10^4 \pm 8.99 \times 10^3$ copies per ng of total RNA at 15 days post-infection (dpi) and undetectable at 30 dpi. ITS transcript levels in trout skin were at maximum 15 dpi for all *P. salmonis*. For instance, the load of ITS transcripts in the skin of Psal-103-infected trout was $5.44 \pm 2.58 \times 10^6$ copies per ng of total RNA at 15 dpi. There were no significant differences in mortality between infection treatments. The cumulative mortality of trout from the negative control group was significantly lower than those from *P. salmonis*-infected trout. The expression of nine immunity-related genes was determined by RT-qPCR in the gills, spleen, liver, muscle, and head kidney tissues. An innate inflammatory response was associated with the expression of the *saa* and *tnf-α* genes in surviving fish. In addition, the downregulation of *oncmk-dbb* and *IGHM* genes indicates that *P. salmonis* can interfere with the activation of CD4⁺ T cells and impair humoral immunity. Our findings suggest that the isolate Psal-104 has a higher immunogenic potential. Finally, our results support the use of non-lethal

and non-invasive methods for analyzing fish skin as an early surveillance tool for piscirickettsiosis.

KEYWORDS

piscirickettsiosis, salmon rickettsial septicemia, reverse transcription-quantitative PCR, immune gene marker, seawater bath challenge model, LF-89-like genotype, EM-90-like genotype, LF-89 genotype

Introduction

The facultative intracellular marine Gram-negative γ -proteobacteria *Piscirickettsia salmonis* is the etiological agent of piscirickettsiosis or salmon rickettsial septicemia (SRS). The disease was first reported in Chile in the 1980s and then during the next years in Canada, Scotland, Ireland, and Norway (1, 2), which is currently the world's largest salmon producer. Even though piscirickettsiosis does not cause significant economic losses in Norway and other salmon-producing countries, it was responsible for ~44.7% of deaths caused by infectious microorganisms in Chilean salmonid farms during 2023 (3). This disease causes economic losses exceeding US\$1,000 million annually for the Chilean salmonid industry (4). Importantly, the disease affects rainbow trout (*Oncorhynchus mykiss*) during the on-growing stage (3), which, in Chile, is carried out in a seawater farming stage. There are currently no effective treatments for piscirickettsiosis, other than antibiotics, with florfenicol being the drug of choice (5). The use of antimicrobial drugs in fish farming is a controversial issue due to the potential impacts on aquatic environments, risks of selecting and spreading drug-resistant bacteria, and increased levels of antibiotic resistance among pathogens (5).

The *P. salmonis* type strain LF-89^T (ATCC VR-1361) was isolated in Chile in 1989 from a piscirickettsiosis outbreak in Coho salmon (*O. kisutch*) farms (6). Chilean isolates of *P. salmonis* have been separately clustered with the LF-89^T and EM-90 prototypes to form the genogroups 1 and 2, respectively (7). A hybrid group among LF-89^T and EM-90 prototypes has also been proposed, in addition to genogroups 3 (LF-89^T-like isolates) and 4 (EM-90-like isolates) (7). A recent study indicated three distinct genogroups for the *P. salmonis* lineage on a global scale (8); that is, the NC genogroup conformed by Norwegian and Canadian isolates, and the aforementioned genogroups 1 and 2. Isolates from genogroups LF-89 and EM-90 exhibit different levels of virulence. EM-90 isolates show increased pathogenicity in post-smolt Atlantic salmon (*Salmo salar*) under *in vivo* conditions (9, 10). Furthermore, salmon infected with EM-90-like isolates can experience higher mortality rates compared to those infected with LF-89-like isolates. In fact, there are studies reporting the full survival of salmon infected with some LF-89-like isolates (11). Research involving new isolates of *P. salmonis* from different genogroups is required to corroborate the previously mentioned patterns. For example, *P. salmonis* Psal-103 and Psal-104 (which are affiliated to LF-89-like and EM-90-like prototypes, respectively) were isolated from Atlantic salmon during piscirickettsiosis outbreaks in Chilean farms. However, only a limited number of *in vitro* studies have explored the potential mechanisms through which these field

isolates cause the disease. The two isolates exhibit adhesion capacity and form biofilms, with Psal-104 biofilms showing greater tolerance to salmon skin mucus (12). This finding indicates that Psal-104 cells may possess enhanced resistance to the innate immune components present in fish, such as mucus lysozyme found in rainbow trout skin mucus (13). Notably, the *in vivo* virulence of both isolates in salmonid species has yet to be assessed.

Fish affected by piscirickettsiosis exhibit clinical signs such as loss of appetite, lethargic swimming, anemia, and pale gills, as well as hemorrhagic lesions of the skin and muscle, intestinal necrosis, and pathological changes in all internal organs (1). The *in vivo* immune response of Atlantic salmon to piscirickettsiosis is well-documented (10, 14), while information on this topic in rainbow trout is less comprehensive (15). This gap in knowledge stands in contrast to the extensive research on the *in vitro* effects of *P. salmonis* on immune gene expression in rainbow trout cell lines (16–19). Differences in host preference (or host range) among *P. salmonis* genotypes could be expected considering the following points: (i) EM-90-like isolates have primarily been recovered from Atlantic salmon, (ii) LF-89-like isolates have been found indistinguishably in both Atlantic salmon and rainbow trout, and (iii) LF-89-like isolates are recovered less frequently from Coho salmon (20). Indeed, factors related to host preference and/or the *P. salmonis* genotype (21) may contribute to variations in fish survival to piscirickettsiosis and in the corresponding immune response.

The first study to provide evidence that *P. salmonis* is primarily acquired from the environment through the skin and gills of rainbow trout, rather than the oral route, was conducted by Smith et al. (22). Currently, there is limited but increasing evidence supporting the idea that the non-injured skin and gills are the main entry points for the bacterium in salmonids (23, 24). Based on this rationale, immersion infection challenge models may more accurately simulate the natural entry route of waterborne pathogens, such as *P. salmonis*, compared to artificially induced infections via intraperitoneal injection. Remarkably, most studies on the fish immune response to microbial infections (including piscirickettsiosis) have relied on intraperitoneal injections. Other studies have used fish cohabitation as a model for the transmission of piscirickettsiosis (10, 14), but this approach overlooks the issue of “specimen zero” in disease outbreaks, as the Trojan fish are also obtained through intraperitoneal injections. It is important to emphasize that emulating more realistic infection settings may be a key issue when evaluating the efficacy of vaccines against piscirickettsiosis.

This study aimed to determine and compare the expression of nine genes involved in the immune response of rainbow trout in the first stage of seawater farming upon *P. salmonis* infections.

The examined genes encode three immune marker groups: (1) cell surface markers and intracellular receptors linked to cell-mediated immunity, (2) components, and (3) modulators of the adaptive and innate immune system. The former group includes genes encoding the cluster of differentiation 4 (CD4-dressed cells within T cell populations that recognize pathogens and secrete cytokines), the major histocompatibility complex class I (MHC I-dressed cells in lymphoid and epithelial tissues present antigens to T cells and natural killer cells) and MHC II (primarily associated with antigen presentation to T cells), as well as the toll-like receptor 3 (TLR-3), which is intracellularly expressed in various tissues (e.g., the liver, pyloric ceca, intestine, spleen, and head and trunk kidney). The second group involves genes encoding the primary systemic antibody immunoglobulin M (IgM) (adaptive component) and the acute-phase reactant serum amyloid A (SAA) (innate component). The third group is represented by genes encoding adaptive/innate modulators, such as pro-inflammatory cytokines (i.e., interleukin-1 β [IL-1 β] and tumor necrosis factor-alpha [TNF- α]) and the anti-inflammatory interleukin-10 (IL-10). In this study, *P. salmonis* infections were induced by seawater baths containing the type strain LF-89^T (genogroup LF-89) and two field isolates, Psal-103 (genogroup LF-89-like) and Psal-104 (genogroup EM-90-like).

Materials and methods

Sources of *P. salmonis*

Employed in this study were the type strain of *P. salmonis*, LF-89^T (genogroup 1), as well as two Chilean bacterial isolates, Psal-103 and Psal-104 (representative of the LF-89-like and EM-90-like prototypes; genogroups 3 and 4, respectively). These field strains were obtained from kidney samples of farmed Atlantic salmon during piscirickettsiosis outbreaks in the Los Lagos Region (Chile). Psal-103 and Psal-104 were included in the present study as both isolates have fully sequenced genomes, and the respective adherence properties and responsiveness to trout skin mucus (as first fish defensive line against waterborne pathogens) have been thoroughly characterized in previous studies (12, 13).

All *P. salmonis* were stored at -80°C in Cryobille tubes (AES Laboratory, France) for long-term storage. All were routinely cultured in solid and liquid AUSTAL-SRS media (25) for 5 d at 18°C and 120 rpm. After bacterial growth, culture axenicity and species identity were confirmed by standard microscopy and PCR targeting a 91 bp fragment of the internal transcribed spacer (ITS) in the ribosomal operon (26).

Rainbow trout immersion infection challenge with *P. salmonis*

Prior to immersion infection challenges, the bacterial concentration (cells mL^{-1}) in cultures was determined on a light microscope at 1,000X magnification using the Breed's slide method and crystal violet dye (27). The number of colony forming units per milliliter (CFU mL^{-1}) was determined through serial dilutions by plating on AUSTAL-SRS agar plates.

Prior to transporting the fish from the fish farm to the experimental facility, 100 rainbow trout (average weight 100 g) were supplied and certified by an external private laboratory, which is validated and officially recognized by the National Fisheries and Aquaculture Service (SERNAPESCA). The certification confirmed that the fish were free of pathogens listed in List 1, as well as endemic pathogens, including *P. salmonis* and other harmful agents such as infectious pancreatic necrosis virus, infectious salmon anemia virus, *Flavobacterium psychrophilum*, *Yersinia ruckeri*, *Renibacterium salmoninarum*, and *Tenacibaculum* species (*T. maritimum*, *T. dicentrarchi*), among others. Upon arrival at CIMARQ, the fish were maintained for acclimation purposes in 600 L continuous-flow aerated seawater tanks for 2 months prior to *P. salmonis* challenges. Seawater was collected at CIMARQ and filtered through a 30- μm sand filter, decanted, and then filtered using cartridge filters (pore sizes: 20, 10, 5, and 1 μm), followed by UV-C irradiation (dose: 40 mJ cm^{-2}). During the acclimatization period, key seawater parameters were monitored and maintained as follows: temperature $14.9 \pm 0.6^{\circ}\text{C}$, salinity $31.34 \pm 1.33\text{‰}$, and dissolved oxygen $8.0 \pm 0.7\text{ mg O}_2\text{ L}^{-1}$. Fish under acclimatization were fed with commercial food pellets at 1.5% body weight daily, subjected to a photoperiod of 12:12 light: dark, and feces were removed every other day.

After acclimation, rainbow trout were divided into five groups, each containing 16 fish. Three groups were separately immersed for 2 h in plastic tanks containing a mixture of 40 L seawater (treated as previously mentioned) and 4 L of a pure culture of each *P. salmonis* to achieve a final concentration of 1×10^7 bacteria mL^{-1} . Fish into this mixture were kept with constant aeration to provide a dissolved oxygen concentration of $\sim 8.2\text{ mg L}^{-1}$. Another group of 16 fish was exposed for 2 h to a mixture of sterile AUSTAL-SRS broth (without *P. salmonis*) and seawater, in the same proportion and under the same aeration conditions as used in the immersion infection challenges. This treatment is hereinafter referred to as “the sterile medium treatment” and was included to assess any influence of fish handling during the immersion infection challenges on trout gene expression patterns. In addition, a negative control group of 16 fish was used for comparison purposes.

Following exposure, fish from each group were transferred to a recirculating aquaculture system (RAS) facility and distributed among 100 L plastic tanks (work volume 80 L) at a stocking density of approximately 12 kg m^{-3} , that is, 8 fish per tank. Therefore, the study was conducted in duplicate tanks. Fish were kept with daily feeding at 1.5% body weight, a photoperiod of 12:12 light: dark, and feces were removed every other day. Dead fish were removed as needed. The RAS was maintained for 30 d at $14.5 \pm 1.5^{\circ}\text{C}$, with a salinity of 30–32 ‰, and constant aeration to provide a dissolved oxygen concentration of $7.5 \pm 0.6\text{ mg L}^{-1}$. Mortalities were sampled by AUSTAL-SRS agar streaking of skin lesions and internal organs (i.e., spleen, liver, head kidney, and gills). The resulting isolates were analyzed via conventional PCR using a primer pair targeting *P. salmonis* (Table 1) (26).

Randomly selected rainbow trout were collected from duplicate RAS tanks for each *P. salmonis* infection group (i.e., Psal-103, Psal-104, and LF-89^T) at 15 and 30 days post-infection (dpi). Fish collection was performed in triplicate, ensuring balanced sampling across the two RAS tanks per group. Specifically, at 15 dpi, two fish were collected from tank 1 and one fish from tank 2 for each

TABLE 1 Characteristics of the primer sets used to amplify interest genes from cDNA templates.

Primer name	Description	Target gene	Sequence (5' to 3')	Target species	Amplicon size (bp)	Melting temp. (°C)	Efficiency (%)	References
CD4_F	Cluster of differentiation 4	<i>cd4-1</i>	CATTAGCCTGGGTGGTCAAT	<i>O. mykiss</i>	89	55	88.2	(29)
CD4_R			CCCTTTCTTTGACAGGGAGA					
MHC I_F	Major histocompatibility complex class Ib	<i>Onmy-IB Mhc</i> region: <i>onmy-UCA</i>	TCCCTCCCTCAGTGTCT	<i>O. mykiss</i>	73	55	98.7	(30)
MHC I_R			GGGTAGAAACCTGTAGCGTG					
MHC II_F	Major histocompatibility complex class II beta-chain	<i>oncmk-dbb</i>	CCACCTGGAGTACACACCC	<i>O. mykiss</i>	117	60	82.6	(31)
MHC II_R			TTCCTCTCAGCCTCAGGCAG					
IgM_F	Immunoglobulin M heavy chain	IGHM	CTTGGCTTGTGACGATGAG	<i>O. mykiss</i>	72	54	80.7	(29)
IgM_R			GGCTAGTGGTGTGAATTGG					
IL1β_F	Interleukin-1 beta 1	<i>il1b1</i>	ACATTGCCAACCTCATCATCG	<i>O. mykiss</i>	91	59	80.3	(32)
IL1β_R			TTGAGCAGGTCCTTGTCTTG					
IL-10_F	Interleukin 10	<i>il10</i>	CGACTTTAAATCTCCCATCGAC	<i>O. mykiss</i>	70	54	83.4	(29)
IL-10_R			GCATTGGACGATCTCTTTCTTC					
TLR-3_F	Toll-like receptor 3	<i>tlr3</i>	CTTCAACAGCCTCACCA	<i>O. mykiss</i>	82	56	80.9	(31)
TLR-3_R			CTCGTTGTGCTGTACGGT					
TNF-α_F	Tumor necrosis factor alpha	<i>tnf-α</i>	TCTTACCGCTGACACAGTGC	<i>O. mykiss</i>	130	57	84.6	(33)
TNF-α_R			AGAAGCCTGGCTGTAAACGA					
SAA_F	Serum amyloid A	<i>saa</i>	GGAGATGATTCAGGGTTCCA	<i>O. mykiss</i>	78	55	94.3	(33)
SAA_R			TTACGTCCCCAGTGGTTAGC					
RTS1_F ^(*)	Internal transcribed spacer in the ribosomal operon	ITS	TGATTTTATTGTTTAGTGAGAATGA	<i>P. salmonis</i>	91	50	97.2	(26)
RTS2_R ^(*)			AAATAACCCTAAATTAATCAAGGA					

Forward (F) and reverse (R) primer names, target genes, primer sequences, target organism, size of the PCR products, melting temperatures (Tm) for annealing, amplification efficiency, and references. ^(*) This primer set was also used for conventional PCR from DNA templates.

infection group, whereas at 30 dpi, one fish was collected from tank 1 and two fish from tank 2 for the same infection group. Various samples were obtained from these fish (see details below) to compare with samples collected from fish in the sterile AUSTRAL-SRS medium treatment and from the pre-challenge condition. For the sterile medium treatment, three rainbow trout, two fish from tank 1 and one from tank 2, were collected 2 h after exposure to the treatment. The pre-challenge condition was established by collecting three rainbow trout directly from 600 L continuous-flow acclimation tanks approximately 2 h prior to the start of the immersion infection challenges.

Collected fish were euthanized using benzocaine at a concentration of 30 mg L⁻¹. Subsequently, skin swabbing was performed using GenoTube swabs (Applied Biosystems) along with tissue sampling from various organs (i.e., gills, spleen, liver, muscle, and head kidney). Skin swabbing was performed by rolling a swab along the lateral line of the fish, from the operculum to the tail, following a previously described methodology (28). Swab samples were stored at -80 °C until total RNA extractions for further analysis. Tissue samples were preserved in RNeasy Lysis Solution (Qiagen, Hilden, Germany) and stored at -20 °C until total RNA extraction for RT-qPCR assays.

Additionally, triplicate 60 mL seawater samples were collected from the RAS tanks at 15 and 30 dpi, as well as from acclimation tanks at days 0 and 30 of the challenge trials. These samples were filtered using sterilized syringes with sterile 25 mm Swinnex filter holders fitted with PVDF filters (0.22 µm pore size, 25 mm diameter; GVWP02500, Millipore). The PVDF filters were air-dried, placed into sterile cryovials containing 300 µL of RNeasy Lysis Solution, and stored at -20 °C until further analysis.

Total RNA extraction and synthesis of complementary DNA (cDNA)

Tissue samples were processed for total RNA extraction using the TRIzol™ Isolation kit (Thermo Fisher Scientific, NY, USA) with an initial tissue disruption step performed using a TissueLyser II (Qiagen, Hilden, Germany). Briefly, 20 mg of tissue were transferred to cryotubes containing a 1:1 mixture of 1.0 and 2.3 mm diameter zirconium beads (Low Binding Zirconium Beads, OPS Diagnostics, Lebanon, NJ) and 1 mL of TRIzol™. The TissueLyser II was operated for 2 × 1.5 min at a frequency of 30 Hz, with a 1 min pause between runs. Following cell disruption, the homogenate was transferred to fresh Eppendorf tubes, and total RNA was extracted according to the manufacturer's protocol for the TRIzol™ reagent. RNA extraction from skin swab samples followed the same mechanical disruption procedure, but exclusively used 200 µm diameter zirconium beads (OPS Diagnostics). Subsequently, microbial debris was transferred to fresh Eppendorf tubes for RNA extraction using the Ambion® RNA extraction kit (AM1560, Ambion, Austin, TX, USA), following the manufacturer's instructions. For total RNA extraction from filtered water samples, the filters were gradually thawed on ice and processed using the same Ambion® kit, incorporating a mechanical disruption step with 200 µm diameter zirconium beads and the TissueLyser II, set for 2 × 1 min at a frequency of 27 Hz. All

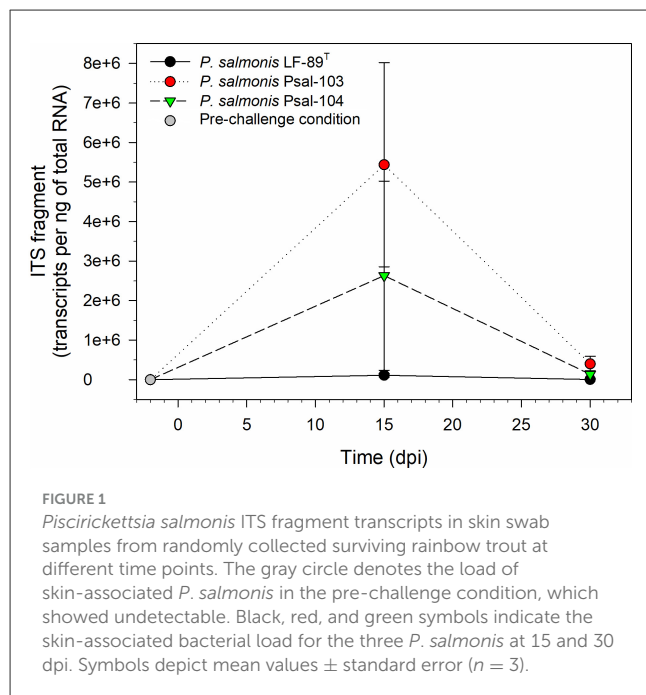
total RNA preparations were resuspended in DEPC-treated Milli-Q water. The concentration and integrity of all RNA extracts were assessed using a Qubit 4 fluorometer (Thermo Fisher Scientific, MA, USA), employing the Qubit™ RNA HS or BR assays and IQ assay kit, respectively (Thermo Fisher Scientific). The average integrity and quality (IQ) value of the total RNA extracts was 8.7 ± 0.5. These samples were stored at -20 °C until further analysis.

To avoid DNA contamination, total RNA samples were treated with the TURBO DNA Free™ kit (Applied 500 Biosystems, Austin, TX, USA), following the manufacturer's instructions. Subsequently, cDNA was synthesized from DNA-free RNA using the ImProm-II™ Reverse Transcription System (Promega, WI, USA), according to the manufacturer's protocol. Reverse transcription reactions were performed as per the manufacturer's guidelines by using 20 ng of DNase-treated RNA and either the Oligo(dT)₁₅ primer set (for host gene expression analysis) or random primer set (for *P. salmonis* load detection), which are commonly used for cDNA synthesis from mRNAs with and without poly(A) tails, respectively. The resulting cDNAs products were quantified using the Qubit™ ssDNA assay kit (Thermo Fisher Scientific) and stored at -20 °C for RT-qPCR analysis.

Two-step RT-qPCR assays

The expression of nine immune-gene markers (Table 1) was quantified by qPCR from cDNA templates derived from RNA extracted from tissue samples, using specific primer sets (29–33). Similarly, transcript levels of a fragment of the ITS region in the ribosomal operon of *P. salmonis* (Table 1) were quantified from cDNA synthesized from rainbow trout skin swab samples. Primer sets were initially selected from the current literature (Table 1). The specificity of the chosen primers and expected amplicon sizes were evaluated using *in silico* tools available through NCBI's Primer BLAST. Only primer sets that passed this preliminary *in silico* analysis were synthesized by Integrated DNA Technologies (IDT). Before performing RT-qPCR assays, we performed *in vitro* validation of the primers via conventional PCR amplification followed by agarose gel electrophoresis. This allowed us to optimize melting temperatures and confirm the size and specificity of the amplicons.

Conventional PCR reactions were performed in a total volume of 25 µL containing 100 ng of cDNA template and the GoTaq® Green Master Mix (2X, Promega, Madison, WI, USA). The thermal cycling program consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of: denaturation at 94 °C for 30s, annealing at the temperature specific for each primer pair (Table 1) for 30s, and extension at 72 °C for 30s. The program concluded with a final extension at 72 °C for 5 min. The GeneRuler Low Range DNA ladder (Thermo Fisher Scientific) was included in every agarose gel electrophoresis (2.5% w/v). Subsequently, selected amplicons generated by conventional PCR were excised from 2.5% (w/v) agarose gels using the Wizard™ SV genomic DNA purification kit (Promega), following the manufacturer's instructions. The concentration of each purified PCR product was measured using the Qubit™ dsDNA HS (Thermo Fisher Scientific). These purified PCR amplicons were used as templates

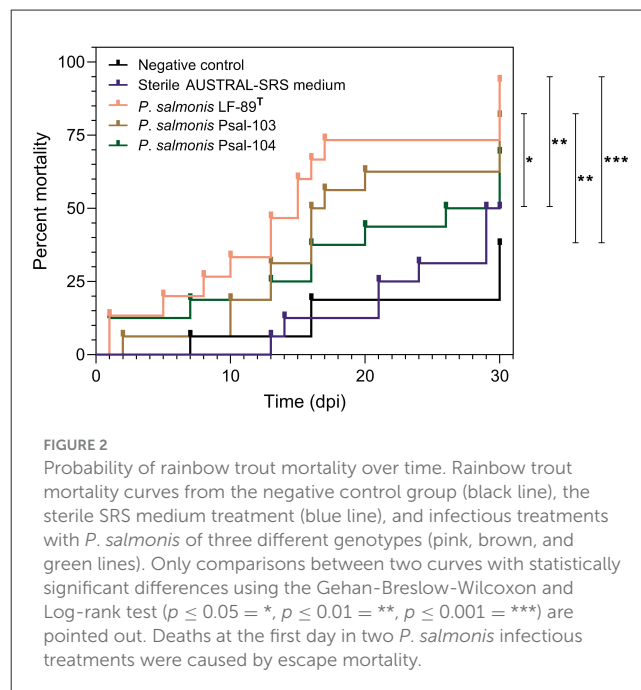


to generate standard curves for qPCR quantification, following a method previously applied in *P. salmonis* studies (34). To calculate amplicon copy number, the concentration ($\text{ng } \mu\text{L}^{-1}$) was divided by the amplicon's molecular mass, estimated using the formula: $\text{mass} = [(n) \times (M/\text{NA})] \times 10^9$ where 'n' is the amplicon length (bp), M is the average molecular weight of a base pair (660 g mol^{-1}), NA is the Avogadro's number ($6,0221 \times 10^{23} \text{ bp mol}^{-1}$), and 10^9 is the conversion factor from grams to nanograms. A six-point standard curve, starting from 4×10^7 copies and prepared through serial 10-fold dilutions, was included in each qPCR run. The qPCR efficiencies and correlation coefficients (R^2) for immunity-related genes were $85.9 \pm 6.1\%$ and 0.96 ± 0.08 (mean $\pm$ SD), respectively. For the *P. salmonis* ITS fragment, efficiencies were $97.2 \pm 0.56\%$, with an R^2 of 1.0 ± 0.0 (Table 1). RT-qPCR cycle threshold (Ct) values for target genes are depicted in Supplementary Figure 1.

The qPCR thermal cycling protocol began with an initial denaturation at 95°C for 3 min, followed by 40 amplification cycles. Each cycle consisted of a denaturation at 95°C for 30 s, annealing for 45 s at a temperature specific to the primer set used (Table 1), and extension at 72°C for 45 s. All reactions were performed using a Stratagene Mx3000P real-time PCR system (Agilent Technologies-Stratagene). The specificity of the resulting PCR products was confirmed by agarose gel electrophoresis and melting curve analysis, which was conducted using MXPro software (version 4.10; Agilent Technologies).

Statistical analysis

The number of gene-transcript copies was calibrated by the total RNA concentration per sample to addresses potential troubles raised during RNA preparation (e.g., RNA quantity and integrity).



Calibrated gene-transcript copies were used to calculate the $\log_2$ -fold change ($\log_2\text{-FC}$) in gene expression between rainbow trout from infectious treatments (with different *P. salmonis*) at 15 and 30 dpi and those collected from the pre-challenge condition, or between *P. salmonis*-challenged trout at 15 and 30 dpi and those collected from the sterile medium treatment. Differential gene expression analysis was performed using the "DESeq2" package and the DESeq function (35). Immune gene markers with statistically significant $\log_2\text{-FC}$ values (i.e., adjusted P-values [$P\text{-adj}$] ≤ 0.05), and thresholds of ≥ 2 and ≤ -2 were considered up- and downregulated, respectively. $\log_2\text{-FC}$ data and respective statistical significance were visualized using the Pheatmap package (v1.0.12) in R (36). Additionally, cumulative mortality curves of rainbow trout were generated from data obtained from seawater immersion infection challenges using the GraphPad Prism 10.4.1 software (GraphPad Software, Inc., CA, USA). Gehan-Breslow-Wilcoxon and Log-rank (Mantel-Cox) tests were used to assess the statistically significant differences between resulting curves ($P \leq 0.05$).

Results

Detection of *P. salmonis* during early piscirickettsiosis and rainbow trout survival

ITS transcripts of *P. salmonis* were undetectable in skin swab samples from rainbow trout in the pre-challenge condition (Figure 1). These transcripts were also undetectable in seawater samples collected from acclimation tanks at days 0 and 30 of the challenge trials, as well as in skin samples from rainbow trout in the sterile medium treatment, which were collected 2 h after exposure to the treatment. The number of ITS transcripts in RAS seawater samples was $2.21 \times 10^4 \pm 8.99 \times 10^3$ gene copies per

ng of total RNA at 15 dpi, but transcripts were undetectable at 30 dpi. *P. salmonis* transcripts were also undetectable in non-skin tissues of randomly selected surviving rainbow trout after the bath challenges, except in some mortalities from the infectious treatments, as detected by conventional PCR at 30 dpi (Supplementary Figure 2). Maximum loads of skin-associated *P. salmonis* in surviving rainbow trout were detected at 15 dpi, but this remarkably decreased at 30 dpi, especially for *P. salmonis* Psal-103- and Psal-104-challenged trout (Figure 1). At both 15 and 30 dpi, the *P. salmonis* load (measured as ITS transcripts of the bacterium) in rainbow trout skin, in decreasing order of abundance, was as follows: Psal-103 > Psal-104 > LF-89^T. For instance, at 15 dpi, the bacterial load of *P. salmonis* was $5.44 \pm 2.58 \times 10^6$ and $1.12 \times 10^5 \pm 4.71 \times 10^4$ copies per ng of total RNA in the skin of Psal-103- and LF-89^T-infected trout, respectively (Figure 1). For comparison purposes, at 30 dpi, the load of *P. salmonis* ITS transcripts in the skin of Psal-104-caused mortalities reached $7.45 \times 10^7 \pm 4.0 \times 10^6$ copies per ng of total RNA.

There were no significant differences in mortality between rainbow trout from the negative control group and those from the sterile medium treatment (Figure 2). Similarly, no significant differences were observed among the infectious treatment groups (Figure 2). The percent mortality in the negative control group was significantly lower ($P \leq 0.05$, Gehan-Breslow-Wilcoxon and Log-rank tests) than in the LF-89^T- and Psal-103-infected groups (Figure 2). Fish from the sterile AUSTRAL-SRS medium treatment also showed a significantly lower cumulative mortality ($P \leq 0.05$, Gehan-Breslow-Wilcoxon and Log-rank tests) compared to the LF-89^T- and Psal-103-infected rainbow trout (Figure 2). Although not statistically significant, cumulative mortality in the negative control and sterile medium groups tended to be lower than in the Psal-104-infected group (Figure 2).

Differential expression of immune gene markers

Log₂-FC values were calculated to identify significant variations in the expression of immune marker genes between the sterile medium treatment and the pre-challenge condition. The null hypothesis asserts that, in the absence of any effects from fish handling, no significant differences in gene expression between these two conditions are expected. Therefore, genes with log₂-FC values between ≤ 2 and ≥ -2 , with at a P-adj value of ≤ 0.05 , were considered to have not changed significantly. These cutoffs were met for some of the tested genes, depending on the tissue sample. Specifically, this was true for the gills (all genes), head kidney (*onmy-UCA*, *oncmk-dbb*, and *tlr3*), liver (*IGHM*, *oncmk-dbb*, and *tlr3*), muscle (*IGHM* and *oncmk-dbb*), and spleen (all genes except *saa*) (Figure 3). In addition, among the genes with log₂-FC that fell within these threshold values (i.e., ≤ 2 and ≥ -2), only those with significant log₂-FC values of ≥ 2 or ≤ -2 , when comparing infectious treatments to the pre-challenge condition, were associated with the differential expression caused by *P. salmonis* (see heat map cells with thick outlines in Figure 4).

Immune markers that showed significant levels of gene regulation induced by *P. salmonis* included *onmy-UCA* and *saa*.

These genes were significantly upregulated in the gills of surviving trout, and *onmy-UCA* was also upregulated in the spleen and head kidney (Figure 4). By contrast, the genes *oncmk-dbb* and *IGHM* were significantly downregulated in the liver and muscle, along with *tlr3* in the liver (Figure 4). In addition, the expression of *tnf-α* varied in the spleen depending on the strain and the post-infection time. This gene was significantly upregulated at 15 dpi in the spleen of LF-89^T-infected surviving rainbow trout; however, in trout infected with Psal-103 and Psal-104, *tnf-α* was significantly downregulated (Figure 4). Additionally, there were interesting findings regarding gene upregulation that could not be exclusively attributed to *P. salmonis*. This was observed in the spleen, liver, muscle, and head kidney for *saa*, as well as for *cd4-1* and *tnf-α* in the liver and muscle of surviving trout (Figure 4).

Discussion

RT-qPCR analysis and other cDNA-dependent approaches are often used for studying the active fraction of predominant bacterial populations by detecting sequence transcripts (37–39). In the present study, *P. salmonis* was detected by RT-qPCR in seawater samples collected from the pilot-scale RAS at 14.5 ± 1.5 °C at 15 dpi, but not at 30 dpi. A prior study used ITS DNA-based qPCR to analyze the water column of aquaculture settings in southern Chile (40); this study detected *P. salmonis* up to 30 days (but not more than 40 to 50 days) after cage emptying. In addition, *P. salmonis* has been reported to survive extracellularly in seawater at 15 °C for approximately 12 days (41). Therefore, although *P. salmonis* can persist in aquaculture settings for at least 30 days after the removal of affected fish, the presently obtained results indicate that only shorter periods ensure the detection of its potential activity by RT-qPCR outside hosts. These results suggest that a potential interference of free-living *P. salmonis* in the pilot-scale RAS was unlikely or minimal.

Piscirickettsia salmonis was detected in skin samples from randomly collected rainbow trout at 15 and 30 dpi (Figure 1), but not in non-skin tissues from these fish, except in some samples randomly collected from mortalities at 30 dpi, and that were analyzed by conventional PCR (Supplementary Figure 2). These observations indicate that the applied sampling strategy, focused on the analysis of samples taken from surviving fish, allowed the collection of data in the first stages of piscirickettsiosis in rainbow trout. The load of skin-associated *P. salmonis* in rainbow trout varied from undetectable in the pre-challenge condition to detectable levels at 30 dpi, with a peak at 15 dpi (Figure 1). In the absence of data on the load of *P. salmonis* in rainbow trout challenged with the bacterium, studies conducted in other salmonid species can be used for comparison purposes. For instance, the load of rainbow trout skin-associated *P. salmonis* in the present study was in the range reported (between 30 and 65 dpi) in the muscle of Atlantic salmon, but compared with head kidney samples (42), the load of trout skin-associated *P. salmonis* was higher in the current study. In addition, the load of *P. salmonis* in the head kidney of Atlantic salmon parrs has been determined by counting of ITS transcripts (43), with results following a bell-shaped pattern between 7 and 42 dpi, with peaks between 13 and 21 dpi. When considering the pre-challenge condition, the

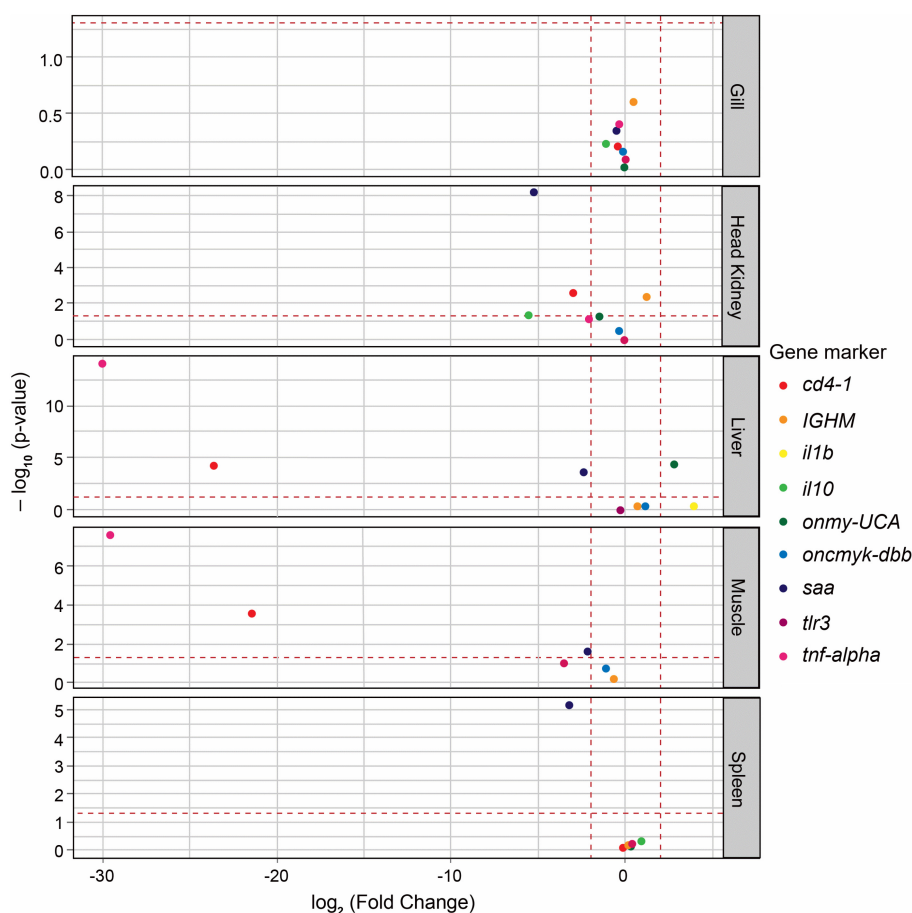


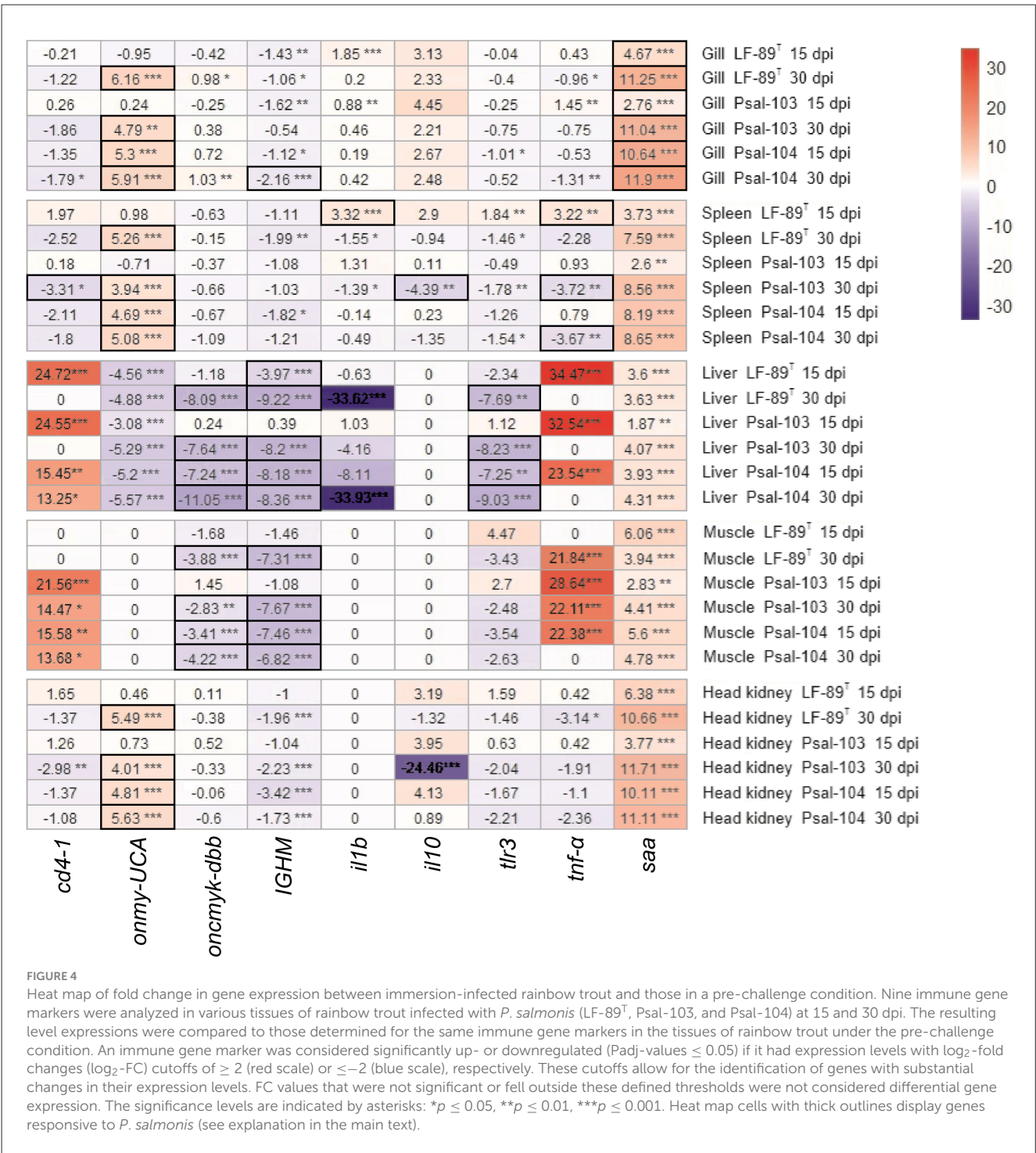
FIGURE 3

Volcano plot displaying the changes in immune gene expression ($\log_2$ -FC) between the sterile medium treatment and the pre-challenge condition. The fold change (FC) values for all genes are plotted on the X-axis, while the negative logarithmic adjusted p -values (Padj) are shown on the Y-axis. The horizontal dashed line denotes the cutoff value of 0.05 for the negative logarithmic Padj-value. The two vertical dashed lines denote the cutoff value of -2 and 2 for the $\log_2$ -FC. Each immune gene is represented by a differently colored circle. Genes located within the range of -2 to 2 and with Padj values of ≤ 0.05 were considered to show no significant variation between the compared conditions.

present study also reports that the load of trout skin-associated *P. salmonis* appears to follow a bell-like shape trend. However, it would be necessary to increase the sampling frequency during the initial post-challenge hours to confirm this trend. The observed decrease in skin-associated *P. salmonis* load toward the end of the study may reflect bacterial clearance from fish. The clearance process has been described in salmonids and represents a key and complex aspect of the disease's natural history. This process can be modulated at multiple interconnected levels, including the participation of non-specific antibodies (44) and autophagy (45). At the skin level, bacterial clearance may occur through secretion of mucus, which traps and sheds microbes. Moreover, rainbow trout skin mucus contains several antibacterial components from both the innate and adaptive immune responses (46, 47), contributing to the clearance processes.

Rainbow trout from the negative control group and sterile medium treatment exhibited the lowest mortalities (Figure 2). Over time, these fish exhibited hyperactive behaviors, contrasting with the lethargy and loss of appetite observed in infected fish. The hyperactivity was linked to aggressive social interactions, such

as caudal fin biting or nipping, which could have contributed to the mortalities in the control group and sterile medium treatment. Most of the recorded mortalities started from day 7 onward (Figure 2). A prior study on *P. salmonis*-challenged rainbow trout by immersion baths recorded the first mortalities around day 10, leading to cumulative mortalities of 91.7% at 23 dpi (48). Rainbow trout challenged by intraperitoneal injection of *P. salmonis* experienced mortalities from day 4 onward, continuing up to 33 dpi (22). Mortality percentages of rainbow trout infected with *P. salmonis* LF-89^T, Psal-103, and Psal-104 did not show significant differences (Figure 2). In other salmonids such as Atlantic salmon, an EM-90-like isolate provoked a higher cumulative mortality in shorter infection times compared to an LF-89-like isolate (9). Moreover, Atlantic salmon might survive challenge trials with some *P. salmonis* LF-89-like isolates but not with EM-90-like isolates (11). However, another study conducted in Atlantic salmon has found no differences in cumulative mortality (or time to mortality) when comparing the effects of LF-89-like and EM-90-like *P. salmonis* isolates (49). Our study likely required a larger sample size to detect potential differences in



mortality among genotypes. However, the lack of differences in mortality between infectious treatments does not necessarily imply that gene expression responses were identical. For instance, the isolate Psal-104 (EM-90-like genotype) tended to induce higher cumulative gene regulation levels over time compared to the other two *P. salmonis* (Supplementary Figure 3). This pattern was observed regardless of whether the gene regulation response met the established criteria for reliable association to solely *P. salmonis* (Figure 3). This pattern aligns with a previous study that compared the effects of LF-89-like and EM-90-like *P. salmonis* isolates on gene expression in post-smolt Atlantic salmon, reporting an exacerbated immune response in PS-EM-90-infected fish, the isolate of higher pathogenicity (10). Although only a few studies have investigated the virulence mechanisms of the Psal-103 and Psal-104 isolates (12, 13), current evidence suggests that the EM-90-like isolate (Psal-104) has a greater potential capacity to overcome the trout mucosal barriers (13).

Genes encoding immune markers such as CD4, IL-1 β , and IL-10 did not appear to show a direct responsiveness to *P. salmonis* in the tested tissues (Figure 4). The gene *tlr3* was often downregulated in various organs (particularly in the liver), or it did not show differential expression (Figure 4). This results aligns with the primary role of TLR-3 in the antiviral immunity of rainbow trout (50, 51), rather than in defending against bacterial infections (52). However, bacterial pathogens like *Aeromonas salmonicida* can also cause the downregulation of the TLR-3-encoding gene in various tissues of rainbow trout compared to controls (53). While TLR-3 is generally considered less important for recognizing bacterial ligands, with only a few examples in fish immunology literature suggesting otherwise (54), the immune response to *P. salmonis* deserves further investigation. For instance, the risk of secondary infections may increase due to the downregulation of TLR-3 caused by *P. salmonis*. This is particularly concerning in the context of emerging infectious diseases, as it could increase the susceptibility of rainbow trout to marine viruses affecting multiple organs, especially the liver.

The *oncmk-dbb* gene, which encodes MHC-II, showed regulation levels that varied from non-differentially expressed to being downregulated in the present study (Figure 4). It was significantly downregulated in the liver and muscle of *P. salmonis*-infected trout, which was accompanied by a significant upregulation of *cd4-1* (a T lymphocyte gene marker) in these tissues (Figure 4). In Atlantic salmon, *P. salmonis* LF-89-like and EM-90-like isolates also reduced the expression of a MHC-II-encoding gene (10). The present study suggests that *P. salmonis* manipulates the activation of CD4+ T cells to escape the host immune response, as CD4+ T cells may be present but cannot detect antigen-presentation cues. Indeed, *P. salmonis* is known to evade the immune system of salmonids by downregulating MHC-II on the surface of infected cells, leading to reduced antigen presentation to CD4+ T cells and, consequently, preventing their activation (1). However, differences in *cd4-1* expression observed between the pre-challenge condition and sterile medium treatment (Figure 3) suggest that the potential proliferation of CD4+ T cells in the liver and muscle was possibly not induced by *P. salmonis* alone. Pathogenic infections are a major influencing factor of CD4+ T cell proliferation in fish, but they are not the only one. For example, the interaction between a fish's immune system and its microbiota can activate the innate immune response, which may promote the proliferation and activation of CD4+ T cells (55). Thus, the presence of *P. salmonis* along with changes in fish's commensal microbiota potentially induced by the sterile medium treatment, could have been responsible for *cd4-1* overexpression in both the liver and muscle.

The *IGHM* gene remained predominantly downregulated in all tissues, indicating that the IgM-based humoral immune response may not have been induced by *P. salmonis*. This response was perceived especially in the liver and muscle (Figure 4). Currently, there is limited information regarding the expression of IgM-related genes in rainbow trout infected by *P. salmonis*. A previous study reported reduced expression of a gene encoding IgM in the head kidney of Atlantic salmon infected by LF-89-like and EM-90-like *P. salmonis* isolates, particularly in fish infected with PS-EM-90 (10). This study also indicated that fish infected by PS-LF-89 showed higher variability in MHC-I gene regulation

(with increased expression at 56 dpi in survivor fish), whereas PS-EM-90-infected fish showed higher expression levels from 35 dpi onwards. In the present study, the expression of the *onmy-UCA* gene, which encodes MHC-I, varied across different tissues. In the muscle and liver, *onmy-UCA* exhibited responses ranged from non-differential expression to downregulation after infections. In contrast, in the gills, spleen, and head kidney, this gene was often upregulated, which suggests an adaptive immune surveillance involving presenting antigens to CD8+ T cells to identify and eliminate *P. salmonis*-infected cells. Although a gene marker for CD8+ T cells was not analyzed, the increased TNF- α levels in rainbow trout's liver and muscle could stimulate T cell maturation in the thymus, potentially including CD8+ T cells (56).

P. salmonis triggers the overexpression of *il10* in the rainbow trout macrophage-like cell line (RTS11) during the early stages of infection. This overexpression helps prevent the inflammatory response and was associated with pathogen survival within macrophages (16). In the current study, however, we did not observe an anti-inflammatory response based on the overexpression of *il10* due to *P. salmonis*. In addition, the gene expression levels of *saa* and *tnf- α* indicated an inflammatory response, but with no involvement of the pro-inflammatory *il1b1* gene. A high-frequency sampling approach could have allowed us to detect *il1b1* expression during the initial hours post-challenge, as demonstrated by studies on *Y. ruckeri*-infected rainbow trout (57, 58). In the present study, the innate inflammatory response was found to be influenced not only by pathogenic stressors but also by other sources of activation. This is suggested by considering the upregulation of the *saa* gene in the spleen, liver, muscle, and head kidney, as well as the increased expression levels of the *tnf- α* gene in the liver and muscle. Indeed, the expression of the *saa* gene in rainbow trout has already been associated with both pathogenic stimuli, such as whole cells and their virulence macromolecules, and non-pathogenic stimuli, such as heavy metals and confinement (59, 60).

Conclusions

We conducted seawater immersion bath challenges using three distinct genotypes of *P. salmonis* to investigate the early stages of piscirickettsiosis in rainbow trout over a 30-day period. The analysis of the surviving rainbow trout after the bath challenges indicated that genes associated with immune markers, such as CD4, IL-1 β , IL-10 and TNF- α , showed minimal response (in gene regulation) to infections caused by *P. salmonis*. Overall, our findings suggest an innate inflammatory response in surviving fish that was related to the infection by *P. salmonis*, but also to non-pathogenic stimuli. The inflammatory response was associated with the expression of *saa* (e.g., in the gills) and *tnf- α* genes (in the liver and muscle), but with no involvement of the pro-inflammatory *il1b1* gene. The downregulation of *oncmk-dbb* and *IGHM* genes indicates that *P. salmonis* can interfere with the activation of CD4+ T cells and impair humoral immunity in surviving fish. However, further research is needed to determine whether the response of the gene *onmy-UCA* in *P. salmonis*-infected rainbow trout was related to the transcriptional activation of CD8+ T cell gene markers. Notably, while there were no significant differences in mortality

among the three *P. salmonis* infectious treatments, the isolate Psal-104 (an EM-90-like genotype) tended to induce higher cumulative levels of gene regulation over time compared to the other two genotypes. In turn, this suggests that Psal-104 may have a greater immunogenic potential. Finally, since the bacterium was found in the skin of the challenged surviving trout but not in their internal organs, our findings support the use of non-lethal and non-invasive analysis of fish skin for the early surveillance of piscirickettsiosis.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Ethics Committee of Universidad Andrés Bello under approval accreditation 001/2025, in addition to being conducted in accordance with previously approved protocols by the institutional committee on ethics, care and use of laboratory animals (CIECUAL, for its acronym in Spanish) of the Universidad de Talca under folio number 2020-06-A. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HL: Funding acquisition, Conceptualization, Writing – original draft, Investigation, Project administration, Validation. HW: Writing – review & editing, Investigation, Methodology. FB-H: Investigation, Writing – review & editing. FP-S: Writing – review & editing, Formal analysis, Methodology. RI: Investigation, Writing – review & editing. RA-H: Conceptualization, Investigation, Funding acquisition, Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This research was funded by Grants FONDECYT Iniciación 11200708 and FONDAP-INCAR 1523A0007 from the Agencia Nacional de Investigación y Desarrollo (ANID, Chile). The APC was partially funded by

Programa de Apoyo para el pago de Procesamiento de Artículos 2025, Dirección General de Investigación, Universidad de Playa Ancha, Chile.

Acknowledgments

We thank Catalina Salinas for her valuable assistance in laboratory experimental procedures.

Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2025.1660383/full#supplementary-material>

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