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Immunological response of the Sub-Antarctic Notothenioid fish *Eleginops maclovinus* injected with two strains of *Piscirickettsia salmonis*

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ABSTRACT

Eleginops maclovinus is an endemic fish to Chile that lives in proximity to salmonid culture centers, feeding off of uneaten pellet and salmonid feces. Occurring in the natural environment, this interaction between native and farmed fish could result in the horizontal transmission of pathogens affecting the aquaculture industry. The aim of this study was to evaluate the innate and adaptive immune responses of *E. maclovinus* challenged with *P. salmonis*. Treatment injections (in duplicate) were as follows: control (100 μL of culture medium), wild type LF-89 strain (100 μL, 1×10^8 live bacteria), and antibiotic resistant strain Austral-005 (100 μL, 1×10^8 live bacteria). The fish were sampled at various time-points during the 35-day experimental period. The gene expression of TLRs (1, 5, and 8), NLRCs (3 and 5), C3, IL-1β, MHCII, and IgMs were significantly modulated during the experimental period in both the spleen and gut (excepting TLR1 and TLR8 spleen expressions), with tissue-specific expression profiles and punctual differences between the injected strains. Anti-*P. salmonis* antibodies increased in *E. maclovinus* serum from day 14–28 for the LF-89 strain and from day 14–35 for the Austral-005 strain. These results suggest temporal activation of the innate and adaptive immune responses in *E. maclovinus* tissues when injected by distinct *P. salmonis* strains. The Austral-005 strain did not always cause the greatest increases/decreases in the number of transcripts, so the magnitude of the observed immune response (mRNA) may not be related to antibiotic resistance. This is the first immunological study to relate a pathogen widely studied in salmonids with a native fish.

1. Introduction

Fish are the first vertebrates to present an immune system similar to mammals, with lymphoid organs that include the thymus, spleen, and kidney, but lacking bone marrow and lymph nodes [1]. As in mammals, the immune system in fish is divided into innate (nonspecific) and acquired (specific) responses, both of which are composed by humoral and cellular components [2]. The cells and molecules of the innate immune system use non-clonal pattern recognition receptors, including C-type lectin receptors, Toll-like receptors (TLRs), and NOD-like receptors (NLRs) [3]. This is in contrast to cells of the adaptive immune system, which express clonal receptors able to specifically recognize antigens and their derived peptides [3]. Both systems communicate through soluble mediators known as cytokines, which mediate the immune response and alert the recognition of a pathogenic stressor, rapidly controlling pathogen growth, promoting inflammation, and triggering an adaptive immune response [3].

Various infectious agents can stimulate the immune system, including Gram negative bacteria such as *Piscirickettsia salmonis*. This facultative intracellular bacterium [4–8] is the etiological agent of Piscirickettsiosis, a systemic disease that colonizes several salmonid organs, including the kidney; liver; spleen; heart; skeletal muscle; brain; intestine; ovary, and gills [9–11]. The most prominent microscopic lesions occur in the liver, kidney, spleen, and gut [12,13].

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Piscirickettsiosis was initially reported as a salmonid disease, although there is evidence that this disease can occur in non-salmonid species, such as *Dicentrarchus labrax* [14], *Atractoscion nobilis* [15], *Oreochromis mossambicus*, and *Sarotherodon melanotheron* [16]. Furthermore, genetic material of this bacterium has been detected in fish native to Chile, including *Eleginops maclovinus*, *Odontesthes regia*, *Sebastes capensis*, and *Salilota australis* [17].

P. salmonis, a bacterium responsible for significant economic losses, can infect, survive, replicate, and disseminate within host monocytes/macrophages without inducing the cytopathic effect [18]. This bacterium can induce the apoptosis of infected salmonid macrophages *in vitro* during the early, intermediate, and late phases of infection [19]. In particular, *P. salmonis* can modulate the expression of genes involved in the innate and adaptive immune responses in the *Salmo salar* head kidney [20,21]. Between 2 and 14 days post-infection with *P. salmonis*, *S. salar* overexpresses genes involved in the inflammatory and oxidative responses and under-expresses genes involved in the adaptive immune response, the G protein signaling pathway, and apoptotic processes [22,23].

Immunological studies evaluating how the tissues of native fish respond to the presence of *P. salmonis* are scarce, mainly in native fish that normally lives in proximity to salmonid culture centers. *E. maclovinus*, known as the Patagonian blenny, is a Sub-Antarctic Notothenioid of the Eleginopidae (Osteichthyes) family, Notothenioidei suborder. The Patagonian blenny is endemic to Patagonia, in South America, and is one of the most eurythermal, euryhaline, and stenobathic representatives of the suborder [24,25]. *E. maclovinus* subsists off of unconsumed pellet feed and salmonid excrements [26]. These feeding behaviors are indicative of a native-farmed fish interaction in the natural environment. One consequence of this interaction would be a transference of microorganisms (e.g., *P. salmonis*) with different degrees of pathogenicity and antibiotic resistance. In Chile, Piscirickettsiosis is controlled primarily through antimicrobial agents and vaccines [13]. Unfortunately, existing treatments are inefficient and frequently lead to the emergence of antibiotic-resistant isolates [27].

The role that native fish play in transmitting Piscirickettsiosis remains unknown, although *E. maclovinus* increases serum immunoglobulin levels in response to an injected extract of total *P. salmonis* proteins [28]. Additionally, Martínez et al. [29] reported that *E. maclovinus* injected with live *P. salmonis* modulate the genic expression of ferritin-H, suggesting the possible activation of nutritional immunity in the presence of this bacterium [30].

The objective of this study was to evaluate the temporal activation of the innate and adaptive immune responses in two poorly investigated tissues of *E. maclovinus*. Spleen and gut tissue, as well as serum, were obtained after *E. maclovinus* was challenged with two *P. salmonis* strains: the LF-89 reference strain (ATCC® VR-1361™) and the antibiotic-resistant Austral-005 strain. Both strains exhibit genomic differences that would seem to determine different degrees of virulence or pathogenesis [27,31].

2. Materials and methods

2.1. Samples

The present study used the same specimens and experimental procedures as in Martínez et al. [29,30]. Briefly, immature *E. maclovinus* (20 ± 5 g body weight) were captured and transferred to the Calfuco Coastal Laboratory (Faculty of Sciences, Universidad Austral de Chile, Valdivia, Chile). Fish were acclimated (four weeks) in 500 L tanks with seawater (32 psμ, 1085 mOsm kg^{-1}), at a density of 3.1 kg m^{-3}, using a flow-through system, natural photoperiod, and a temperature of 12.0 ± 0.5 °C, following indications given in Vargas-Chacoff et al. [32]. Fish were fed once daily with a 1% body weight ratio of commercial dry pellets (Skretting Nutrece 100 ML; containing 48% protein, 22% fat, 13.5% carbohydrates, 8% moisture, and 8.5% ash). All experiments were performed in compliance with guidelines established by the Comisión Nacional de Ciencia y Tecnología de Chile (CONICYT) and the Universidad Austral de Chile for the use of laboratory animals.

2.2. Primers design

All steps involving massive cDNA sequencing were performed at the Austral-omics Laboratory on a GS Junior Titanium Series (Roche) following manufacturer protocols. In detail, total RNA was extracted from one tissue (head kidney of a healthy fish) with the commercial RNA NucleoSpin® RNA Kit (Macherey-Nagel). RNA was selected with optimal integrity (RIN > 7). Subsequently, mRNA isolation was performed with the PolyATtract III Kit® in an mRNA Isolation System (Promega). The quantity and quality of total mRNA was evaluated using an A260 ND-1000 spectrophotometer (NanoDropH Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. The cDNA library was constructed following the "Rapid cDNA library preparation" protocol recommended by Roche. Bioinformatics analysis of the obtained data included the formation of transcripts (contigs); specifically by using a combination of specialized transcriptomic analysis software followed by transcript annotation against the non-redundant NCBI database. After this process, mRNA sequences of TLR1, TLR8, NLRC3, NLRC5, major histocompatibility complex class II (MHCII), and immunoglobulin M (IgM) were obtained and analyzed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). RNA-seq revealed some genes for the immune response, and the other genes selected for study were amplified from heterologous primers (C3, IL-1β, and TLR5). Genes related to the innate immune response included C3, TLR (1, 5, and 8), NLRC (3 and 5), and IL-1β. Genes related to the adaptive humoral immune response included MHCII and IgMs. Partial cDNA coding sequences were obtained and deposited in GenBank, the accession numbers of which are indicated in Table 1.

2.3. P. salmonis LF-89 and Austral-005

Inoculates of *P. salmonis* were kindly donated by the Laboratory of Metabolism and Biotechnology, Institute of Biochemistry and Microbiology, Faculty of Sciences, Universidad Austral de Chile (Valdivía, Chile). The LF-89 strain was used as a reference [33], and Austral-005 was used as an antibiotic-resistant strain [34]. Both strains of *P. salmonis* were grown in AUSTRAL-TSFe agar plates and incubated at 18 °C for ten days, according to standard conditions [8]. Subsequently, *P. salmonis* colonies were grown in 4.5 mL AUSTRAL-SRS broth at 18 °C for 72 h with moderate agitation (75 rpm) [7].

2.4. Infection assays with P. salmonis LF-89 and Austral-005

After acclimation, *E. maclovinus* specimens were randomly distributed among rectangular tanks (100 L), each of which corresponded to a control/treatment group, in duplicate (n = 126 total). The groups were as follows: (i) Control group, injected only with the culture medium (100 μL, n = 42); (ii) LF-89 Experimental group, injected with the *P. salmonis* LF-89 type strain (100 μL; 1×10^8 concentration of live bacteria, n = 42); and (iii) Austral-005 Experimental group, injected with the *P. salmonis* Austral-005 antibiotic-resistant strain (100 μL; 1×10^8 concentration of live bacteria, n = 42). Six fish per group were collected at 1; 3; 7; 14; 21; 28 and 35 days post-injection (dpi) (i.e., 18 fish total sampled per time-point). Specimens were sampled to evaluate the temporal activation of genes associated with the innate immune system (initial dpi) and adaptive immune system (latter dpi). During the experiment, the fish were maintained at a density of 3.1 kg m^{-3}, temperature of 12.0 ± 0.5 °C, with a flow-through system, and under a natural photoperiod, as per Vargas-Chacoff et al. [32]. Fish were fed once daily at a ratio of 1% body weight using commercial dry pellets (Skretting Nutrece Defense 100). The bacterial dose was the same used in previous studies [29,30].

Table 1
Primer sequences for immune system used in the experiments.

Primer	Nucleotide sequences (5′→3′)	PCR product size	Efficiency Spleen (%)	Efficiency Gut (%)	Accession No. GenBank
MHCII βFw	CTACGAGTTCTACCCCAAACCCAT	102bp	102.38	101.93	MF945987
MHCII βRv	CAGTCGCTGTCAGCCAGTTCTT				
C3 het Fw	ACTGCAGGGAGTCTTTAGATTTG	140bp	101.47	104.24	–
C3 het Rv	TGTGGGCCAGTACTCGATCCAGG				
IL-1β het Fw	TGCAACATGAGCCAGATGTGGA	135bp	102.69	102.01	–
IL-1β het Rv	CGTTGATTCTGAGCCCTTCATCCT				
NLRC3 Fw	TACGATTGCTCCCGTGAAGCTGTT	102bp	102.86	102.01	MF945985
NLRC3 Rv	GTACCGAGCTCTACGCTCACTTTT				
NLRC5 Fw	AGTGATCAATCCGTGGGCGTTT	137bp	101.01	99.66	MF945986
NLRC5 Rv	TCTGGAGTCGAGGCTCATATCAGT				
TLR1 Fw	CAACGCTATCTGATCCCCAAGCAA	114bp	102.47	104.50	c
TLR1 Rv	AAAGCCGACGCTCAGTGTTTGT				
TLR5 het Fw	TGGCTCACTACCAGCTGATGAA	112bp	102.74	102.04	–
TLR5 het Rv	AGCCGCTCATAAAACCACTC				
TLR8 Fw	TCCTGCAGAACTCTCACTTCCT	122bp	104.84	102.73	MF945983
TLR8 Rv	TCTGACCACATTCCTCAGGTTT				
IgMs Fw	TGAAAGACTTCTACCCGCATGAGG	124bp	101.70	104.34	MF945988
IgMs Rv	AACTGGCCATAAGCGGAAAAGG				
18s het Fw	GTCCGGGAAACCAAAGTC	116bp	102.11	103.72	–
18s het Rv	TTGAGTCAAATTAAGCCGCA				

2.5. Sampling procedure

Food was withheld from fish 24 h prior to each sampling time-point. Subsequently, the fish were anesthetized with a lethal dose of 2-phenoxyethanol (1 mL L^{-1}), weighed, measured, and sampled. Blood was collected from capillaries in the caudal region and centrifuged to obtain serum (5 min, 2000 g, 4 °C). The spleen and whole gut were aseptically dissected from each sampled fish and, as with serum, stored at −80 °C until analyses.

2.6. Total RNA extraction

Total RNA was extracted from spleen and gut samples (50 mg each) via homogenization in the TRIzol Reagent (Ambion), following the manufacturer's instructions. The RNA pellets were dissolved in diethyl pyrocarbonate water and stored at −80 °C. RNA was quantified spectrophotometrically at 260 nm (NanoDrop Technologies), and quality was evaluated using electrophoresis on 1% agarose gels. Reverse transcription reactions to synthesize cDNA used total RNA (2 μg) as a template, MMLV-RT reverse transcriptase (Promega), and the oligo-dT primer (Invitrogen), according to standard procedures.

2.7. RT-qPCR gene expression analysis

Reactions were carried out on an AriaMx Real-Time PCR System (Agilent). cDNA was diluted to 100 ng and used as a template for RT-qPCR analysis using the Brilliant SYBRGreen qPCR reagent (Stratagene) and specific primers. Primers were designed for complement component 3 (C3); TLRs 1, 5, and 8; NLRCs 3 and 5; interleukin 1 beta (β); MHCII; IgM; and 18s. Reactions were performed in triplicate in a total volume of 14 μL, containing 6 μL SYBRGreen, 2 μL cDNA (100 ng), 1.08 μL of primers mix, and 4.92 μL of PCR-grade water. The applied PCR program was as follows: 95 °C for 10 min, followed by 40 cycles at 90 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s. Melting curve analysis of the amplified products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. Expression levels were analyzed using the comparative Ct method ($2^{-\Delta\Delta CT}$) [35]. Data were expressed as the fold change in gene expression normalized to an endogenous reference gene (housekeeping18s) and relative to the untreated control. The primers used are listed in Table 1. The PCR products were resolved on 2% agarose gel, purified using the E.Z.N.A® Gel Extraction Kit (Omega Biotek), and sequenced by Macrogen Inc. (South Korea). Sequences were identified using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov) and compared against sequences present in the GenBank database. All data are given in terms of relative expression and are expressed as the mean ± standard error (SE) of the mean. PCR efficiencies were determined by linear regression analysis performed directly on sample data using LinRegPCR [36].

2.8. Serum immunoglobulin

Serum samples were subjected to indirect enzyme-linked immunosorbent assays (ELISA). The ELISA plates were seeded with total protein extracts (1 μg/100 μL^{-1}) of *P. salmonis* for 12 h at 4 °C. Plates were then washed with 0.05% PBS Tween and blocked with BSA for 1 h; after which, serum samples from each fish (1:100) were added and incubated for 2.5 h at 17 °C. After washing, anti-IgM (100 μL; 1:1000) was added to each well and incubated for 1 h at room temperature. The plates were then washed again, and anti-IgG conjugated to peroxidase (100 μL; 1:2500) was added and allowed to incubate for 1 h at room temperature. After incubation, the plates were washed again, and the enzyme substrate (TMB Reagent) was added. The reaction was stopped after 30 min with 2M H_2SO_4. Absorbance was measured at 450 nm on a Synergy 2 plate reader (Biotek) with the Gen5 v1.10 software. The intensity of the color formed was proportional to the concentration of anti-*P. salmonis* salmon IgM in the sample.

2.9. Statistical analyses

Assumptions of variance normality and homogeneity were tested. Data were logarithmically transformed when needed to fulfil conditions for parametric analysis of variance (ANOVA). Two-way ANOVA was performed using the different injections and times as factors of variance, followed by a *post-hoc* Tukey test to identify differences between groups. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Mortality assessments after P. salmonis infection

No mortality or behavioral changes were observed for any group during the experimental period (i.e., 0 to 35 dpi).

3.2. Genes involved in the innate immune response

In the spleen, genic C3 expression in the LF-89 group was

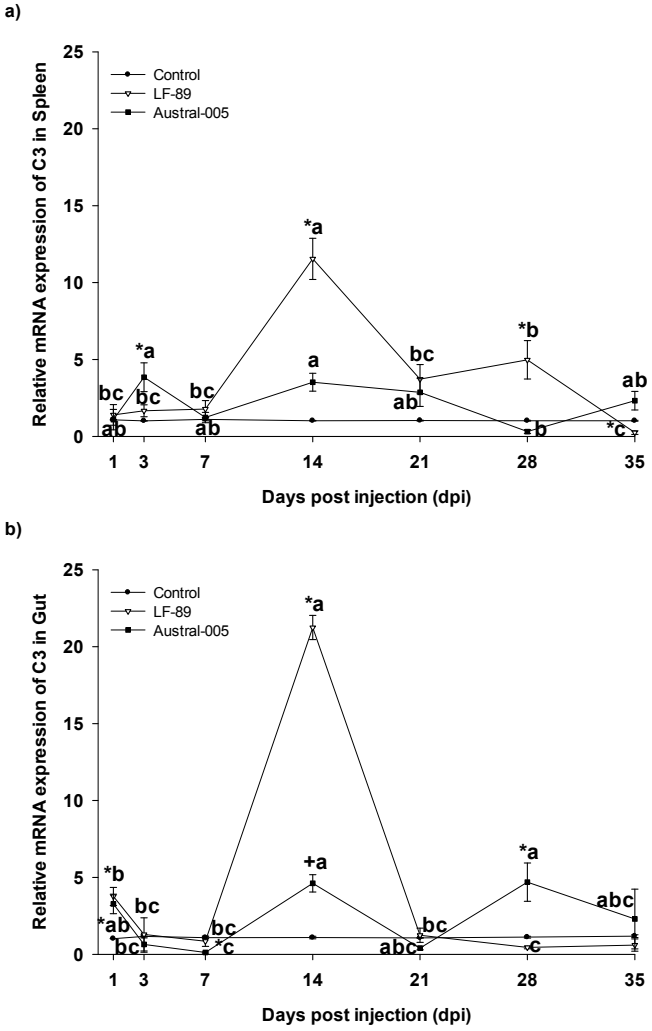

Fig. 1. Gene expression of C3 in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, $P < 0.05$).

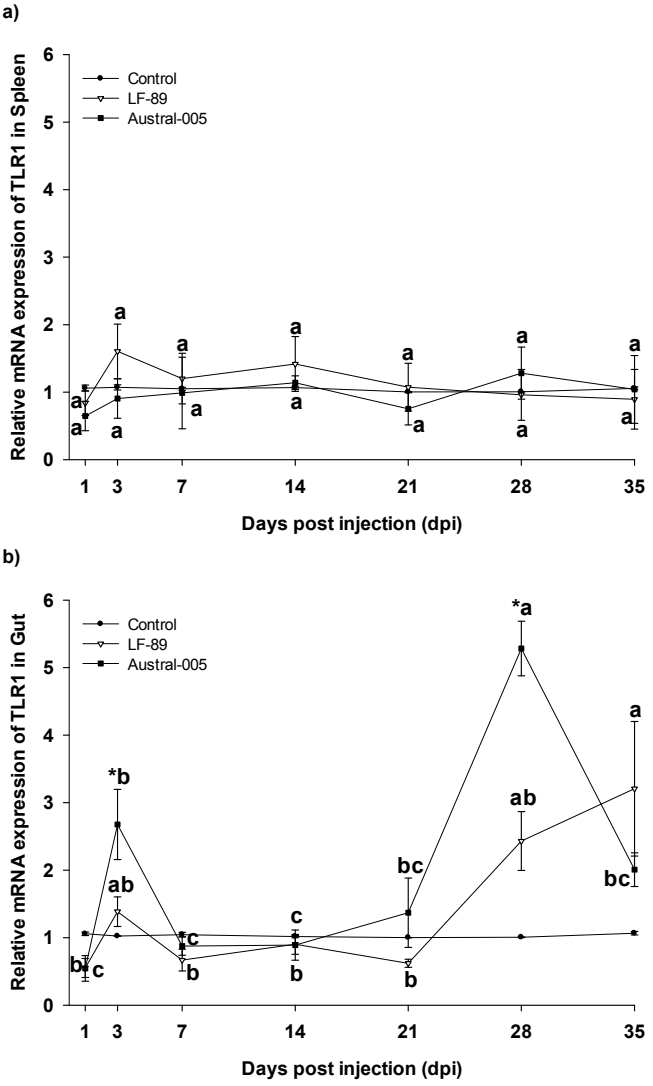

Fig. 2. Gene expression of IL-1β in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, $P < 0.05$).

incremented at 14 and 28 dpi, but decreased at 35 dpi, as compared to the control. The Austral-005 group only exhibited significant differences against the control for C3 expression at 3 dpi (Fig. 1a). In the gut, C3 significantly increased at 1 dpi for both bacterial groups, with peak expressions recorded at 14 dpi for the LF-89 strain (20-fold change) and Austral-005 strain (5-fold change) (Fig. 1b).

The spleen did not present statistical differences in the gene expression of TLR1 (Fig. 2a) and TLR8 (Fig. 3a) over the experimental period. However, gene expression of TLR1 in the gut was upregulated in the Austral-005 group at 3 and 28 dpi (Fig. 2b). The expression profiles of TLR8 in gut were similar for both bacterial strains, showing upregulation at 3, 28, and 35 dpi, but without variations as compared to the control at 1, 7, 14, and 21 dpi (Fig. 3b).

TLR5 expression in spleen was significantly upregulated at 7 and 14 dpi for the LF-89 strain and at 14 and 28 dpi for the Austral-005 strain (Fig. 4a). In the gut, TLR5 expression was statistically upregulated at 14, 28, and 35 dpi in the LF-89 group, while the Austral-005 group showed upregulation at 3, 7, and 28 dpi (Fig. 4b).

In the spleen, genic IL-1β expression in the LF-89 group, as compared to the control, significantly increased at 1, 3, and 14 dpi. The Austral-005 group showed significant IL-1β expression at 1 and 14 dpi. Neither infected group showed differences against the control at 21, 28, or 35 dpi (Fig. 5a). Expression of IL-1β in the gut was statistically upregulated only at 3 dpi for the Austral-005 strain, without significant variations against the control at 1, 7, or 14 dpi. Furthermore, IL-1β was not detected in any group from 21 to 35 dpi (Fig. 5b).

Regarding the expression of NLRCs in the spleen, the expression of NLRC3 was only significantly increased at 14 and 28 dpi for the LF-89 and Austral-005 groups, respectively (Fig. 6a). In the gut, NLRC3 showed highly variable expression over the experimental period, principally in fish injected with the Austral-005 strain. The LF-89 group showed upregulation of NLRC3 expression at 14, 28, and 35 dpi, although levels were not significantly different, compared to the control at 28 and 35 dpi (Fig. 6b).

Finally, NLRC5 expression in the spleen was statistically upregulated at 3 dpi for both treatment groups. In the Austral-005 group, upregulation was again recorded between 21 and 35 dpi. The LF-89 group also evidenced significant upregulation at 35 dpi (Fig. 7a). In

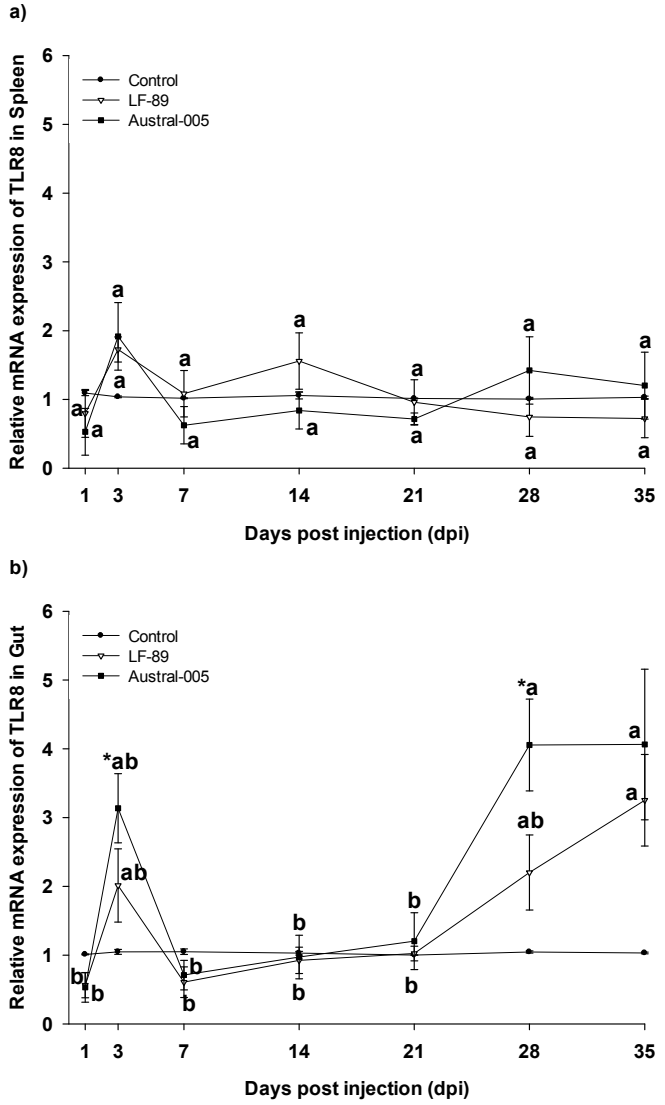

Fig. 3. Gene expression of MHCII in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, P < 0.05).

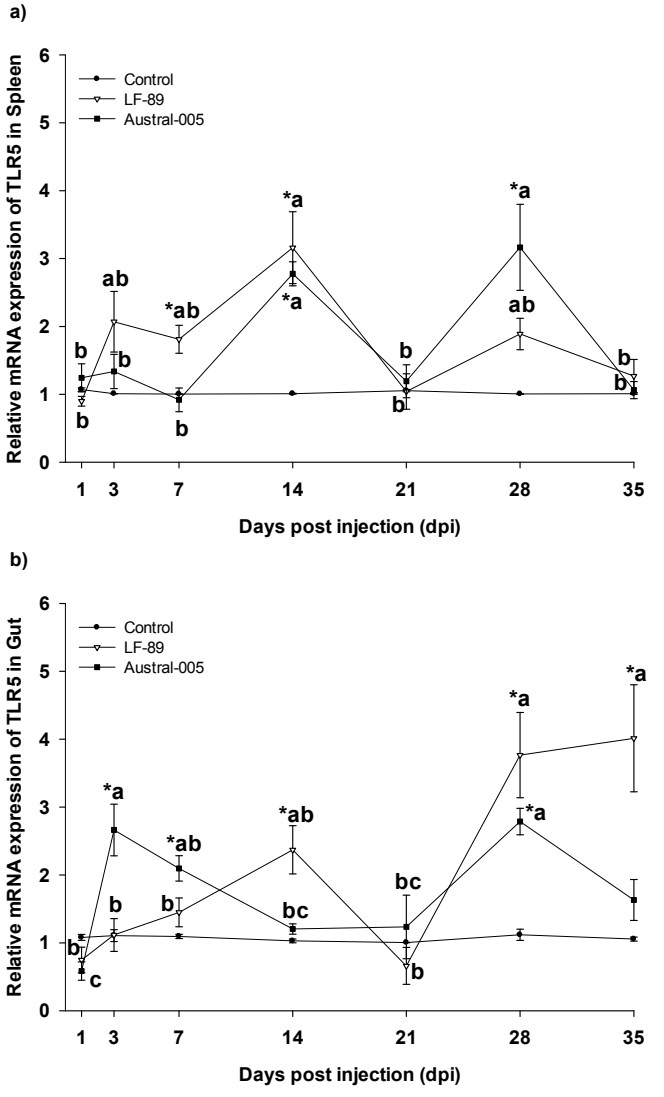

Fig. 4. Gene expression of NLRC3 in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, P < 0.05).

turn, NLRC5 in the gut was positively modulated at 1 dpi for the LF-89 group and decreased at 7 dpi for Austral-005 fish. No statistically significant variations were found for the remaining sampling time-points (Fig. 7b).

3.3. Genes involved in the adaptive immune response

The expression profile for MHCII in the spleen was similar for both evaluated *P. salmonis* strains. In the LF-89 group, MHCII expression was increased at 3, 28, and 35 dpi. In turn, the Austral-005 strain showed downregulated MHCII expression at 1 and 7 dpi, but significantly upregulated expression at 21, 28, and 35 dpi (Fig. 8a). In the gut, MHCII showed peak expression at 1 dpi for both bacterial strains, with the Austral-005 strain again showing upregulated expression of this gene at 28 dpi. However, MHCII expression was decreased at 7, 14, and 35 dpi in the Austral-005 group and at 35 dpi for the LF-89 group (Fig. 8b).

Finally, the splenic expression profile for IgMs was similar in both bacterial groups, with highly upregulated expression (6- to 8-fold change) observed at 3 dpi for the LF-89 and Austral-005 groups as compared to the control. Likewise, expression of this gene remained significantly increased until the end of the experimental period, beginning from 14 dpi for the LF-89 group and from 21 dpi for the Austral-005 group (Fig. 9a). Genic gut expression of IgMs was upregulated at 1 and 28 dpi but downregulated at 7, 21, and 35 dpi following injection of the Austral-005 strain. In turn, the LF-89 strain caused a statistically significant increase in IgMs expression at 3 and 7 dpi, but downregulation at 21 and 35 dpi (Fig. 9b).

3.4. Serum IgM

Levels of the anti-*P. salmonis* antibody in serum did not show statistical differences compared to the control condition between 1 and 7 dpi. However, from 14 to 28 dpi, antibody levels increased in the LF-89 group as compared to the control, while fish injected with the Austral-005 strain maintained increased antibody production from 14 to 35 dpi

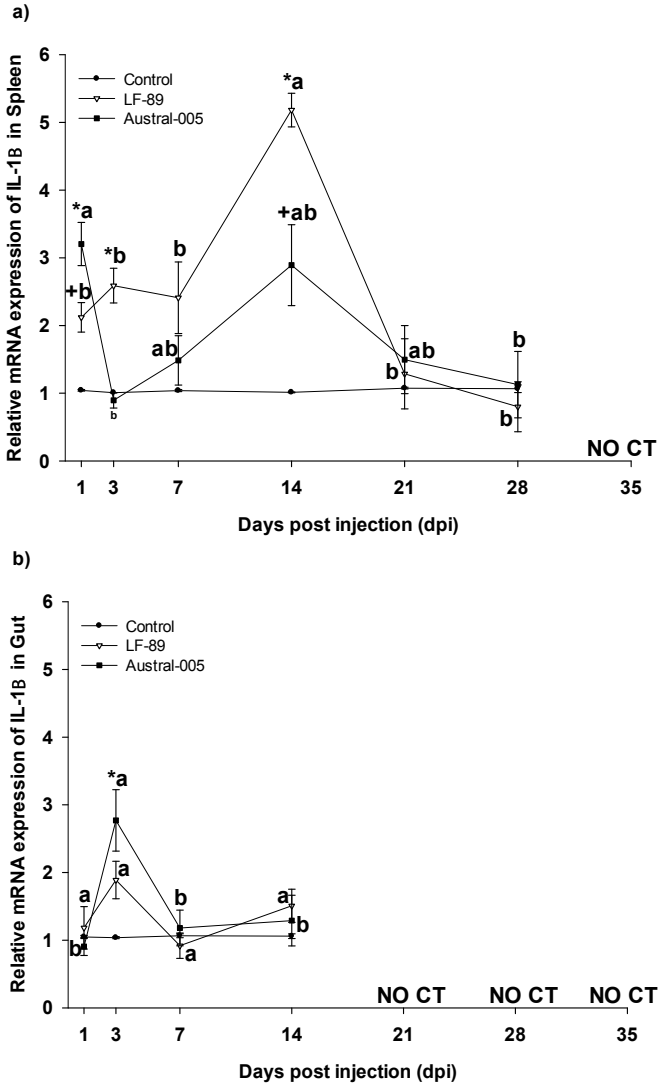

Fig. 5. Gene expression of NLRC5 in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method (2-ΔΔCT) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, $P < 0.05$).

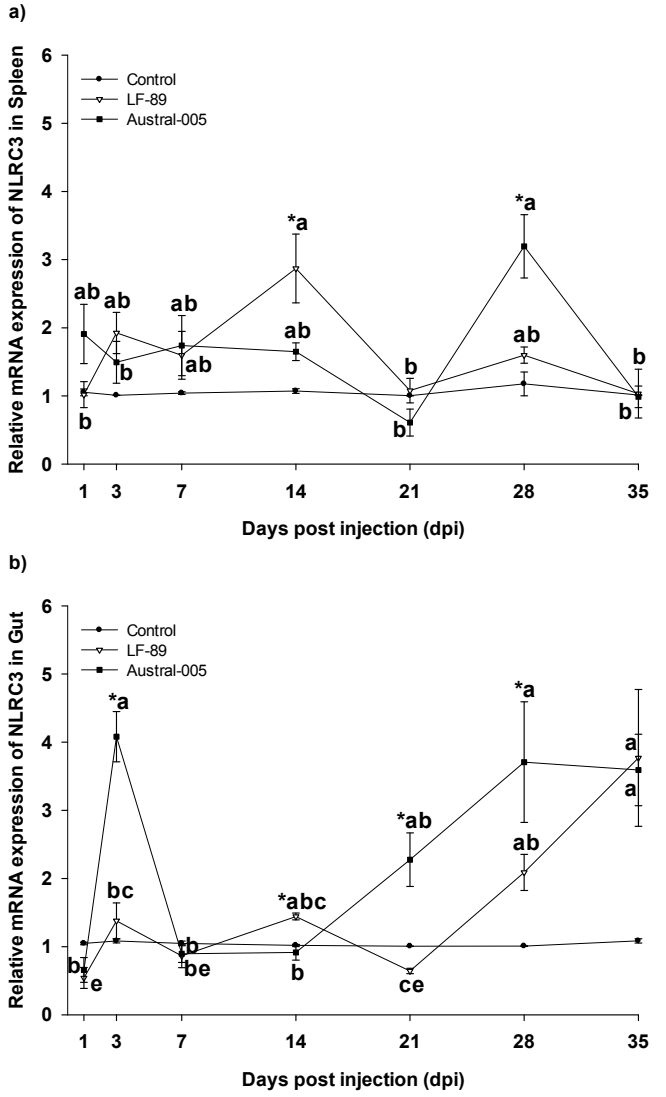

Fig. 6. Gene expression of TLR1 in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, $P < 0.05$).

(Fig. 10).

4. Discussion

Although the immune system has been widely studied in commercially farmed fish, the immune mechanisms of native fish that cohabitate with farmed fish are largely unknown, despite the exchange of pathogen between native and farmed fish. In the present study, transcriptomic analysis was used to obtain partial nucleotide sequences for *E. maclovinus* MHCII, NLRC3, NLRC5, TLR1, TLR8, and IgMs. These genes, together with C3, IL-1β, and TLR5, exhibited modulated expressions when *E. maclovinus* was injected with two strains of *P. salmonis* (excepting TLR1 and TLR8 in the spleen). The profile expressions of MCHII and IgMs in the spleen and of NLRC5 and TLR8 in the gut were similar between both injected strains. Nevertheless, the other evaluated genes showed tissue-specific profile expressions depending on the bacterial strain injected and sampling time-point. The genetic differences between the LF-89 strain [31] and Austral-005 strain [27,34] might explain the differential host responses in modulating specific genes in the spleen and gut.

Published research on the expressional responses of immune genes to *P. salmonis* infection primarily focusses on the anterior kidney of salmonids and in the use of primary cell culture or cell lines [20–23]. Indeed, the present study is the first to provide similar insights into the expressional response of immune genes in the spleen and gut of a native fish species challenged with *P. salmonis*. The conducted challenge assay revealed that the spleen and gut of *E. maclovinus* differentially respond to injections with the LF-89 and Austral-005 strains, evidencing a transcriptional activation of the innate and adaptive immune responses. Immunologically, the fish spleen is a major secondary lymphoid organ that is involved in the presentation of antigens and initiation of the adaptive immune response [37–39]. In turn, the fish gut plays a role in immune homeostasis, protecting against potentially harmful microbes and inducing a tolerogenic response [39].

The gene expression of C3, one protein of the innate immune system, was more elevated in the gut than in the spleen, with

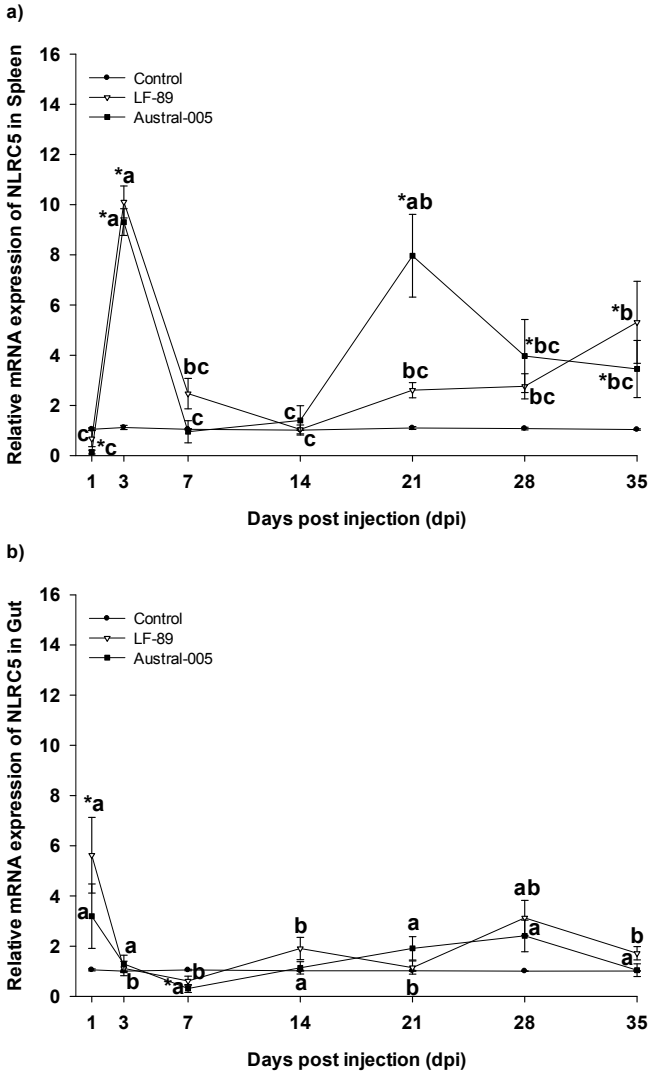

Fig. 7. Gene expression of TLR8 in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, $P < 0.05$).

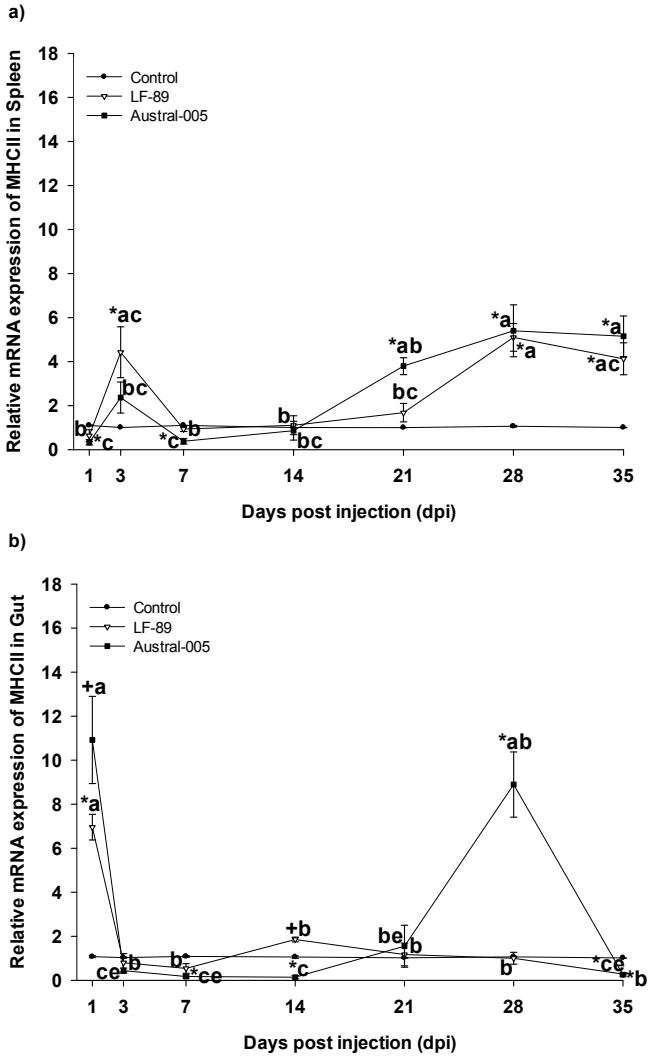

Fig. 8. Gene expression of TLR5 in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenges with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, $P < 0.05$).

differences between the injected bacterial strains. C3, the central component of the complement system, is activated into its respective cleavage products (i.e., C3a and C3b) through three pathways [3]. C3a, also known as anaphylatoxin, is a peptide with multiple functions in the immune response, such as inducing the chemotaxis of eosinophils and mast cells [40–42]; inducing respiratory bursts in eosinophils, neutrophils, and macrophages [43–45]; enhancing phagocytes [46]; and stimulating interleukin-1β expression in monocytes [47]. In turn, C3b is a highly reactive peptide that binds to amino or hydroxyl groups present on microorganism surfaces [3,48]. The recorded increase in C3 transcription following *P. salmonis* injection suggests an activation of both the C3a and C3b immune responses in the spleen and gut.

The NOD-like and Toll-like receptors are pattern recognition receptors that activate the innate immune response following the detection of pathogen associated molecular patterns. These receptors activate the MyD88-dependent or independent signaling pathway, inducing the production of cytokines such as TNF-α, IL-1β, IL-12, IL-8, IL-6, and IFN [49]. In the present study, TLR1 and TLR8 did not evidence significant differences compared to the control treatment in the spleen. In turn, the expressions of these genes in the gut were only statistically increased after injection with Austral-005 strain. The different expression profiles generated in the spleen and gut suggest a tissue-specific modulation of TLR1 and TLR8, which is probably dependent on the function of each receptor and respective specific interactions with each *P. salmonis* strain. TLR1 is located on the cell surface and detects lipopeptides of Gram-positive bacteria [50] and lipopolysaccharides of Gram-negative bacteria [51]. In turn, TLR8 is located within the cell and detects single-stranded viral/bacterial RNA [52]. In other fish, such as *Epinephelus coioides*, *Oplegnathus fasciatus*, and *Pseudosciaena crocea*, the expressions of both receptors are modulated when challenged with different viruses and bacteria [53–56].

On the other hand, TLR5 expression was upregulated in the spleen and gut of *E. maclovinus* during the experimental period for both *P. salmonis* strains. This gene exhibited tissue-specific expression depending on the administered strain, suggesting a possible detection of bacterial flagellin. This flagellin can be found in the membrane of *P. salmonis* [57] and can presumably activate the soluble or membrane

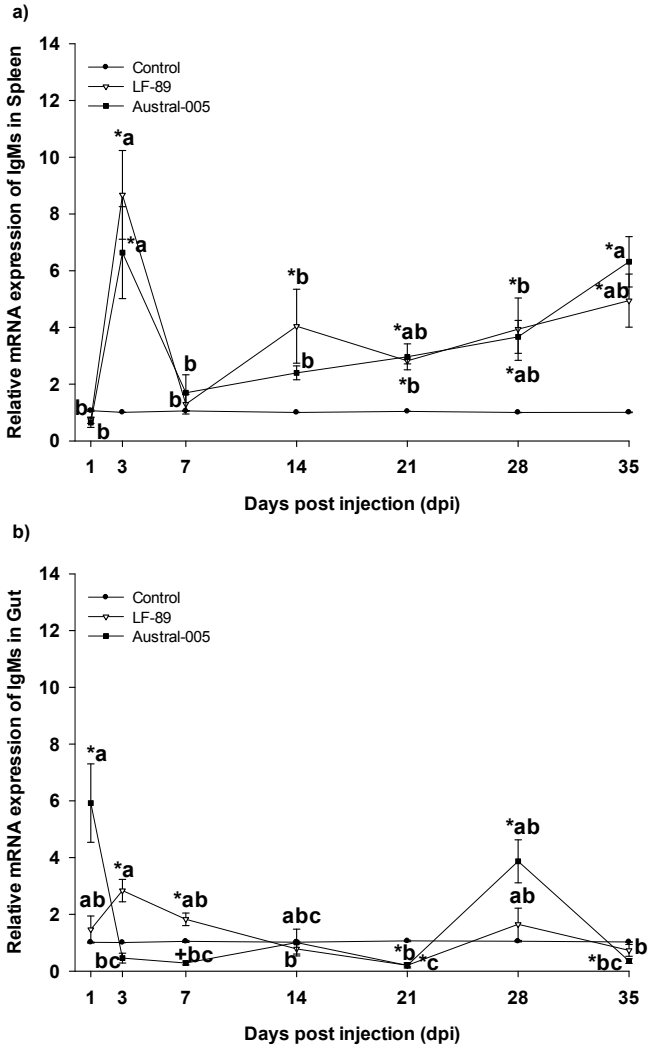

Fig. 9. Gene expression of IgMs in the spleen **(a)** and gut **(b)** of *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) using the 18s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, P < 0.05).

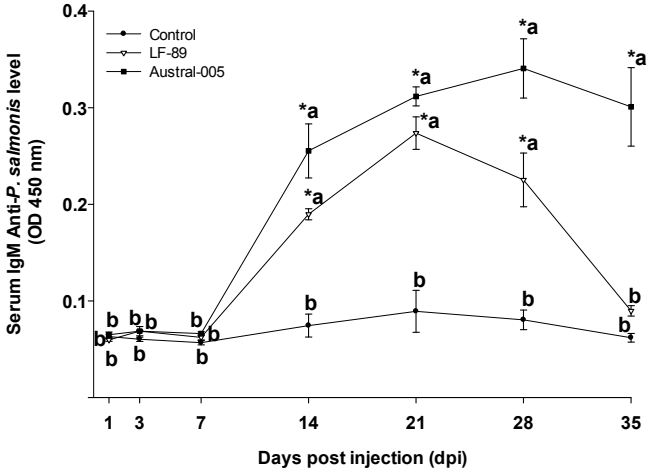

Fig. 10. Total serum IgM levels in *E. maclovinus* specimens challenged with the *P. salmonis* strains LF-89 (▽) or Austral-005 (•) over a 35-day period. Each value represents the mean ± S.E.M. (n = 6). Different letters indicate statistical differences within the same treatment between time-points. Symbols (* and +) indicate statistical differences among different treatments (control, LF-89, and Austral-005) at the same time-point (two-way ANOVA, P < 0.05).

form of TLR5 [58]. *P. salmonis* can modulate the expression of both types of TLR5 in SHK-1 cell lines stimulated with live/inactivated *P. salmonis* or total *P. salmonis* proteins [20].

IL-1β is one of the proinflammatory cytokines of the innate immune system produced by the TLRs pathway of phagocytic cells during the early response to infection or injury [59]. This cytokine is synthesized as pro-IL-1β, an inactivated precursor that must be cleaved by caspase-1 to generate the bioactive, proinflammatory state [60]. In the present study, IL-1β expression evidenced punctual differences between the injected *P. salmonis* strains, with increased expression in the spleen over the course of the experiment, and a slight increase in the number of transcripts in the gut. These data indicate a likely activation of the TLRs pathway and, therefore, the initiation of the immune response post-injection with *P. salmonis*. Furthermore, IL-1β was not detected at 35 dpi in the spleen or from 21 to 35 dpi in the gut, possibly since this cytokine forms part of the early response to infection. In Antarctic fish, such as *Chionodraco hamatus*, this molecule is induced both *in vitro* and *in vivo* after a challenge with lipopolysaccharide [61], and expression is also modulated in the SHK-1 cell line when stimulated with *P. salmonis* [20].

Another group of pathogen recognition receptors are the NOD-like receptors, which are intracellular microbial sensors of the innate immune system [62]. In the present study, NLRC3 and NLRC5 were detected in the spleen and gut of *E. maclovinus*, with tissue-specific expression profiles. Specifically, NLRC3 exhibited punctual modulations in the spleen, whereas NLRC5 expression greatly varied over the duration of the experimental period. The abundance of NLRC3 transcripts increased in the gut during the course of the experiment, particularly for fish injected with the Austral-005 strain. In turn, NLRC5 transcripts in the gut only significantly increased at 1 dpi for both bacterial strains. These results are consistent with reports for other fish species. In *Sebastes schlegelii*, *Ictalurus punctatus*, *Paralichthys olivaceus*, and *Miichthys miiuy*, for example, NLRCs are expressed in all tissues with important immune functions, including the spleen and gut, suggesting important roles in sensing viruses or bacteria [63–66].

The recorded increase in TLR and NLRC transcripts suggests that cellular components of the innate immune system (e.g., monocytes and macrophages) are activated in the spleen and gut as a result of *P. salmonis* injection. These components increase the secretion of IL-1β, thereby initiating the innate immune response and triggering the adaptive immune response [3,67]. In the present study, MHCII gene expression was tissue-specific, with differences between the injected strains in the spleen and gut. The increased number of transcripts would indicate a transcriptional activation of the innate and adaptive immune responses, particularly as MHCII can be found in antigen presenting cells, such as activated macrophages, activated B lymphocytes, and activated dendritic cells [3]. In fish, MHC is ubiquitously expressed in the tissues of healthy individuals, with expressional increases or decreases when host individuals are challenged with bacteria [68–70]. Furthermore, families of *S. salar*, *O. mykiss*, and *Oncorhynchus kisutch* with distinct degrees of susceptibility to *P. salmonis* exhibit allelic differences in MHCI and MHCII, suggesting that specific alleles can be found in families with resistance to this bacterium [71].

In the present study, gene expression of the heavy chain IgMs was detected in the spleen and gut of *E. maclovinus*, with tissue-specific expression profiles that varied depending on the injected bacterial strain. IgMs was significantly modulated in the initial and latter days post-injection. An up-regulated expression of IgMs could indicate a greater formation of μ-type chains and, therefore, an increase in the production of IgM-type antibodies. B cells are activated when the innate immune system is stimulated and through the specific binding of the

epitope to the B cell receptor. This binding event sends a signal to the nucleus through accessory proteins that trigger the crosslinking of B cell receptors to the start of the co-stimulatory signal [3]. Once activated, B cells proliferate and mature to secrete specific immunoglobulins against the detected epitope. In teleosts, these immunoglobulins are termed IgM, IgD, and IgT [72]. IgM^+ B cells comprise the majority of B cells in systemic lymphoid organs, including the head kidney and spleen. IgM^+ B cells also represent the majority of B cells in the blood and peritoneal cavity. In contrast, IgT^+ B cells outnumber IgM^+ cells in the gut, skin, gills, and mucosal-associated lymphoid tissues [72]. In this study, serum antibody levels increased from 14 to 35 dpi for Austral-005 specimens and from 14 to 28 dpi for LF-89 specimens, with no statistical differences between strains, except at 35 dpi, suggesting an activation of B cells through the detection of *P. salmonis* epitopes. The extended increase in serum antibody levels in the Austral-005 group (until 35 dpi) could be related to the greater persistence of this antibiotic-resistant strain [34]. Vargas-Chacoff et al. [28] reported increases in the serum levels of IgM in *E. maclovinus* 14 dpi with total proteins of *P. salmonis*, indicating an activation of the adaptive immune system after bacterial stimulation.

5. Conclusion

This study is the first to report nucleotide sequences for various genes associated with the innate and adaptive immune responses in *E. maclovinus*. Additionally provided is a temporal analysis of respective gene expressions following a challenge with two *P. salmonis* strains presenting distinct degrees of antibiotic resistance. All evaluated genes evidenced increases and decreases in transcript abundances over the course of the experimental period, excepting TLR1 and TLR8 in the spleen. Expression profiles were tissue-specific and showed punctual differences between the injected strains. The Austral-005 strain did not always cause the greatest increases/decreases in the number of transcripts, so the observed magnitude of the immune response may not be related to antibiotic resistance. Expression of the assessed genes was not only detected in two important lymphoid tissues, but also the serum. Specifically, the recorded increased production of anti-*P. salmonis* antibodies in the serum supports a possible activation of the innate (early dpi) and adaptive (latter dpi) immune systems. These data support that *E. maclovinus* tissues respond at the transcriptional level against *P. salmonis*, a pathogen widely studied in salmonids.

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