“DETERMINING THE HEMATOLOGICAL AND IMMUNOLOGICAL PARAMETERS AND MECHANISMS INVOLVED IN HOST RESPONSES TO P. SALMONIS AND CO-INFECTIONS”

This is a working synthesis prepared by Acuaim SpA in order to advise a Consultative Committee, based on the original Report prepared by the Laboratory of biotechnology and aquatic pathology of Austral University, the Clinical Laboratory of Austral University and the Pathovet Laboratory in February of 2017.

PROGRAMA PARA LA GESTIÓN SANITARIA EN LA ACUICULTURA

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ABSTRACT

This work identifies patterns for hematological and immunological parameters in salmonid species challenged with LF-89 and EM-90 P.salmonis strains in intraperitoneal conditions and through cohabitation. Moreover, the mechanisms and pathways modified in fish challenged with pathogenic agents are identified. These results increase the understanding of the different mechanisms through which P. salmonis interacts with the host and they can help with the selection of molecular markers, essential for the improvement of diagnostic techniques, vaccines and therapeutic treatments in aquaculture. In addition, immunity patterns for a PRV coinfection were explored.

INTRODUCTION

In Chile, the SRS control strategy is mainly focused on antimicrobial therapies and vaccinations. However, SRS is the main reason behind the large volume of antibiotics currently being used, and the vaccines are not effective in preventing the disease either, since the accumulated mortality at the end of the cycle does not present any significant differences with respect to the non-vaccinated fish, though they do delay the first outbreak and they reduce its severity. The innate immune system is the first defense line against pathogenic agents, and its main function is to recognize pathogens as early as possible and to activate an adequate pro-inflammatory response. The recognition mechanisms can encourage the direct removal of pathogens by means of phagocytosis or by directing the activation of the adaptive immune response. Fish possess specific immunity mediated by cells characterized by antigen presentation and specificity, by immunological memory and by the participation of lymphocytes T CD4+ and CD8+. The IL-12 encourages transformation into Th1 cells; however intracellular bacteria have developed mechanisms that interfere with the recognition and presentation of antigens, which regulate the evolution of persistent infections. It has been shown that a macrophage in vitro infection of a rainbow trout with P. salmonis induces the overexpression of IL-10 and the subexpression of IL-12, a strategy that inactivates the antibacterial response and facilitates its survival and intracellular replication. The pathogenesis of intracellular pathogenic infections is a continuous battle between the host's defense mechanisms and the bacteria's specific mechanisms for avoiding this response and for facilitating its replication and intracellular survival, including pro-inflammatory cytokines, chemotactic migration of immune cells to the infection site and manipulation of vesicular trafficking mechanisms in order to avoid the toxicity of the lysosomal enzyme and the autophagy pathways.

Concomitant infections can affect the described patterns above and at the same time produce more severe cases of diseases. This is why it is necessary to address various coinfections with P. salmonis and other pathogenic agents present in the Chilean salmon industry at field sample level.

METHODOLOGY

Determining the Reference Intervals (RI) for hematological parameters, blood biochemistry, endocrinology and blood-gas in pre-smolt, smolt, adults and breeders from the three salmonid species farmed in Chile.

This activity is used to determine the reference intervals for hematological biomarkers, gases, hormones and blood biochemistry for the salmonid species farmed in Chile according their productive development phase.

The activity starts with the selection of an animal model according to fish species. A total sampling of 630 healthy specimens from Atlantic salmon, rainbow trout and Coho salmon species were taken, as well as pre-smolt - smolt in freshwater (40-120 g), post-smolt - adults in seawater (0.3 and 2.5 Kg) and breeders in freshwater. Blood samples were also taken and conserved in order to maintain the cold chain (4 – 8°C) up until their analysis in the laboratory. The samples were analyzed for the following parameters:

Blood gas parameters: the analysis was carried out in the field, by means of a portable blood analysis device. The following levels were determined: bicarbonate (HCO_3), total carbon dioxide (TCO_2), blood base excess (BEb) and base excess of extracellular fluid (BEecf).

Hormonal profile: this profile was quantified through the electrochemiluminescence method with automated equipment, following the manufacturer's recommendations. Cortisol (CRT) (ng/mL), thyrostimulant (TSH) (µLU/mL), thyroxine (T_4) (nmol/L), triiodothyronine (T_3) (nmol/L), follicle-stimulant (FSH) (mlU/L), lutenizing hormone (LH) (mIU/L), progesterone (P_4) (ng/mL), estradiol (E_2) (ng/mL), corticotropin (ACTH) (pg/mL), prolactin (PRL) (ng/mL), testosterone (TET) (ng/L).

Blood biochemistry parameters: 23 blood biochemistry parameters were determined, including substrates, enzymes, minerals and electrolytes.

Manual hematology parameters: the following analyses were carried out: Erythrogram (red series), Leucogram (white series) and Thrombocytes (platelet series).

The results obtained from the hematological, blood biochemistry, gas, and endocrinological analyses explained above were analyzed statistically in order to establish RI per salmonid species and productive stages, using a Reference Value Advisor freeware.

Immune response parameters in Atlantic salmon challenged with LF-89 and EM-90 P. salmonis isolates through post-infection and survivor RT-PCR.

The activity describes and comparatively quantifies the expression of genes related with the innate and adaptive immune responses of fish infected with P. salmonis LF-89-like and EM-90-like during the early and late infection phases in cohabitating Atlantic salmon.

The work will be carried out with bacterial strains PS-LF-89 and PS-EM-90, cultivated in Austral-TSHem agar for 6 days at 18°C according to the indications from Yáñez and col (2013). Lastly, an inoculum of 12ml of the isolate PS-LF-89 and PS-EM-90 was obtained with a known DO625 equivalent to 105,6 UFC/ml.

The experimental design considered 5 tanks of 1m3 with a total of 150 Atlantic salmon post-smolts with an average weight of approximately 118,4 g and an 8,4% variation coefficient. The work was done in experimental groups, the first group being intraperitoneally (IP) inoculated with PS-LF-89, together with cohabitants without any inocula. Group 2 was inoculated in the same manner but with the PS-EM-90 inoculum, both groups having an initial infection pressure of 37%.

The test lasted 61 days, and data regarding feeding, mortality, temperature, oxygen and water salinity was registered daily. 5 cohabitant fish were sampled as alive or dying 7, 14, 16, 19, 21, 24, 28, 35, 42, 49 and 56 days after the inoculation (dpi) of the Trojan fish, which were marked.

Samples of the anterior kidney were collected in a RNA later for RT-qPCr analysis in order to evaluate the relative expression of the 16S rRNA gene. At the same time, the following target genes were selected in order to evaluate immunity: IFNγ (infg), TNFα (tnfa), IL-1β (il1b), IL-8 (il8), IL-10 (il10), IL-12β (il12b), IL-15 (il15), IL-18 (il18), major histocompatibility complex class I y class II (mhc1, mhc2), CD4 (cd4), CD8β (cd8b), immunoglobulin M (igm), T-bet (tbx21), Eomesodermin (eomes), perforin 2 (mpeg1) and granzyme A (gzma). The ELF1α and β-actin reference genes were used for calculating relative expression, described by Olsvik and col (2005).

The data was analyzed using descriptive analysis and lineal regression models. The level of statistical significance was established at $p < 0,05$. The correlation between the expression of immune genes and the average load of P. salmonis in the anterior kidney (log10) was explored using descriptive statistics and through the adjustment of lineal or quadratic models for each group of infected fish.

Transcriptomic profile of the immune response in Atlantic salmon challenged with LF-89 and EM-90 P. salmonis isolates through RNA-Seq.

The activity consists of comparatively describing the transcriptional profile of the response in kidneys of Atlantic salmon post-smolt infected with EF-89-like and EM-90-like P. salmonis by means of RNA-Seq sequencing. The transcriptional response was evaluated in the anterior kidney of post-smolt fish from the Trojan Atlantic salmon species (5 days post-inoculation) and in cohabitants (35 dpi) infected with LF-89-like (PS-LF-89) and EM-90-like (PS-EM-90) P. salmonis obtained from an experimental challenge. The samples were deposited in an RNA later up until their extraction, carried out with kits and following the manufacturer's instructions. 5 libraries were compiled using commercial kits. Once the library of clean reads was prepared, the assembly of the de novo transcriptome was carried out using the Trinity v2.0.6 program. The identification of genes was carried out based on the sequences of transcripts assembled using a commercial program. Using these transcripts, we deduced the sequence of amino acids with the help of a program. Following this, the similarity

of the amino acid sequences was calculated compared to a previously calculated available data base. The in silico analysis carried out in order to identify genes with differential expression was carried out with the DESeq2 program, normalizing the count of readings per gene in each condition. In order to validate the reliability of the results of the RNA-Seq, an RT-qPCR was carried out for 12 genes expressed differentially and involved in the immune response, in oxidative stress and in cellular proliferation.

Immune response profiles at transcriptomic level that include innate, acquired and stress immunity, associated with the co-infection in Salmo salar with PRV-ASCV (Piscine OrthoReovirus-Atlantic Salmon Calicivirus), PRV-IPNV (Infectious Pancreatic Necrosis Virus) and PRV-P. salmonis.

A diagnistic of the presence of genetic material of Piscine Orthoreovirus (PRV), Atlantic salmon Calicivirus (ASCV), Infectious Pancreatic Necrosis Virus (IPNV) and P. salmonis was carried out in order to determine the presence or absence of a co-infection with these pathogens. For this, tissue samples maintained in Ethanol and RNA later corresponding to 266 samples from breeders and 80 from freshwater fish of the Salmo Salar species were used. The genes selected for evaluating the immune response in analyzed fish were IFN-I, II, Mx protein, CD4, CD8 and MHC II.

RESULTS

Determining the Reference Intervals (RI) for hematological, blood biochemical, endocrinological and blood-gas parameters in pre-smolt, smolt, adults and breeders for the three salmonid species farmed in Chile.

Reference intervals for blood-gas parameters

No results for oxygen pressure (pO2) and for oxygen saturation (O2Sat) were obtained for any of the specified species, due to the fact that all of the values were lower than the minimum value that this technique can detect.

Table 5. Reference intervals of blood gases for the three salmonid species

Parameter	Unit	Rainbow trout			Atlantic Salmon			Coho Salmon		
		Pre Smolt-Smolt	Post Smolt	Breeding	Pre Smolt-Smolt	Post Smolt-Adult	Breeding	Smolt	Post Smolt	Breeding
pH	mmHg	7,03 - 7,45		7,21 - 7,54	7,13 - 7,46	7,02 - 7,56	7,24 - 7,64		7,28 - 7,54	7,17 - 7,47
pCO_2	mmol/L	14,0 - 24,8		12,0 - 29,8	11,6 - 29,5	11,7 - 20,6	11,2 - 21,9		7,8 - 17,1	16,0 - 25,8
HCO_2	mmol/L	4,9 - 14,2		8,4 - 21,4	8,7 - 14,4	6,7 - 10,5	8,3 - 13,6		5,3 - 9,8	12,6 - 16,7
TCO_2	mmol/L	5,3 - 14,9		9,1 - 23,2	9,5 - 15,1	7,9 - 11,6	8,8 - 14,3		5,6 - 10,3	14,0 - 18,3
Beb	mmol/L	24,3 - 9,7		18,8 - 6,5	20,4 - 9,8	26 - 15,6	18,4 - 9,4		21,8 - 14,5	11,6 - 18,8
Bfecf	mmol/L	26,2 - 10,3		20,4 - 6,5	20,3 - 10,2	28,2 - 17,0	18,3 - 8,4		21,0 - 13,8	12,1 - 19,1

Reference intervals for hormonal parameters

No reference intervals for LH and FSH were obtained for any of the species or productive phases.

Table 6. Reference intervals of hormonal parameter for the three salmonid species

Parameter	Unit	Rainbow trout			Atlantic Salmon			Coho Salmon		
		Pre Smolt-Smolt	Post Smolt	Breeding	Pre Smolt-Smolt	Post Smolt-Adult	Breeding	Smolt	Post Smolt	Breeding
cortisol	ng/mL	8,1 - 98,2	7,0 - 116,6	8,4 - 411,4	2,2 - 102,4	0,4 - 212,6	9,2 - 241,5	37,9 - 162,3	0,8 - 232,6	18,0 - 379,7
TSH	µLU/L	<0,4	*	*	*	<0,02	<0,1	<0,2	<0,1	*
T3	nmol/L	0,7 - 28,5	*	1,8 - 47,8	11,5 - 22,2	24,2 - 62,2	3,3 - 88,8	3,7 - 17,3	1,9 - 22,0	4,9 - 76,5
total T4	nmol/L	5,2 - 11,2	*	5,8 - 72,5	7,0 - 85,4	5,1 - 24,5	3,6 - 38,7	*	-	*
Testostero-ne*	ng/mL	0,01 - 0,3	*	0,04 - 8,4	*	*	*	<2,0	<1,8	*
Progeste-rone*	ng/mL	-	-	0,004 - 0,4	-	-	<0,3	-	-	0,1 - 1,8
Estradiol	ng/mL	<0,13	*	0,1 - 118	*	*	0,03 - 1,3	*	*	0,1 - 24,5
Prolactin*	µLU/L	*	-	*	*	-	<4,9	<12,8	-	*
ACTH	µLU/L	<7,8	*	*	*	*	<2,7	<3,5	*	119,7 - 165,8

TSH: Thyroid-stimulating hormone; T3: Triiodothyronine; T4: Total Thyroxine; ACTH: Adrenocorticotropic Hormone; *Results under the detection limit of the technique in a Cobas e411 equipment.

Reference intervals for biochemical parameters

References ranges for the totality of the biochemical parameters analyzed in the three species were obtained, with the exception of γ-GT which was not detected in any of the species.

Reference ranges for hematological parameters

A table with the hematological parameters was prepared and consolidated, for the three species and the proposed age ranges.

Immune response parameters in Atlantic salmon challenged with LF-89 and Em-90 P. salmonis isolates through RT-PCR.

The results of the relative expression of immunity genes in a P. salmonis infection with LF-89 and EM-90 are presented below.

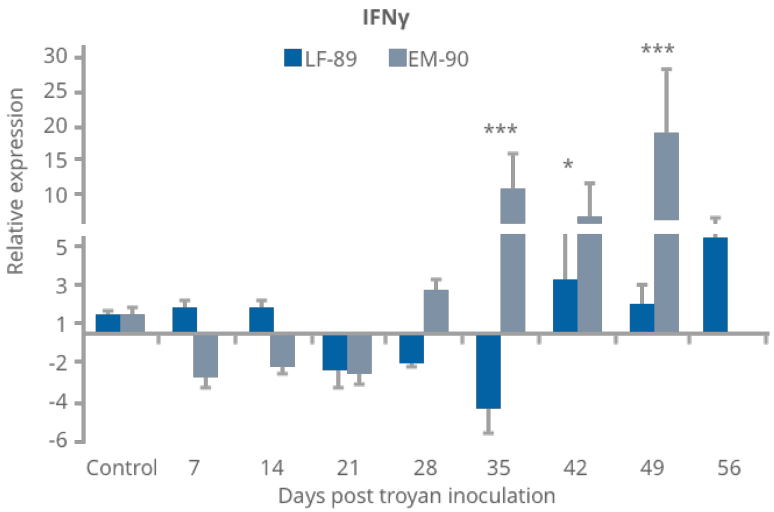

Figure 1: Relative expression of infg in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

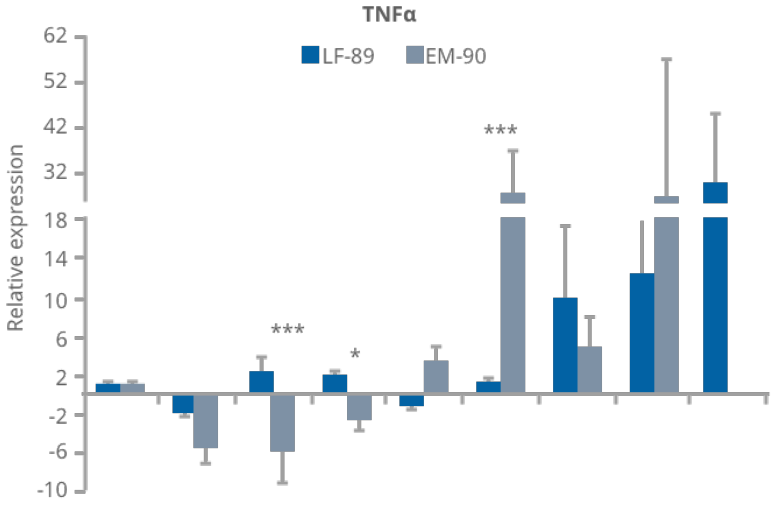

Figure 3: Relative expression of tnfa in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

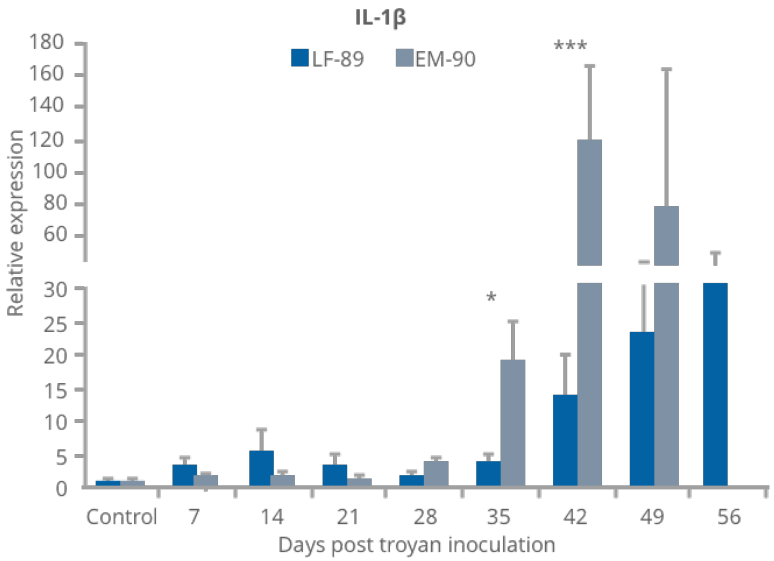

Figure 5: Relative expression of il1b in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

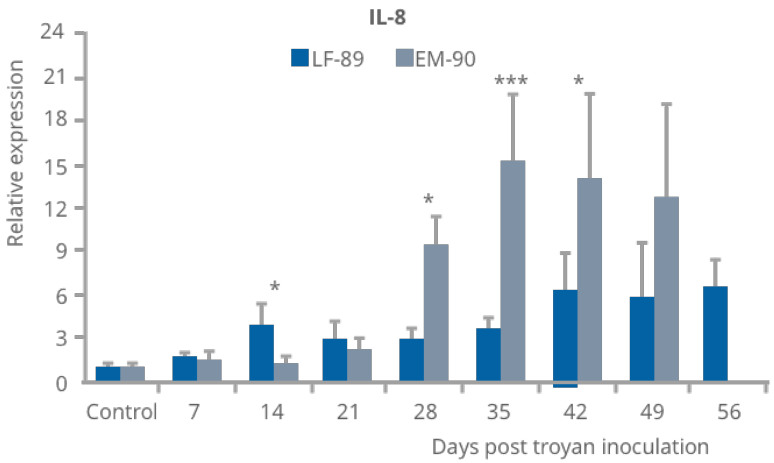

Figure 7: Relative expression of il8 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

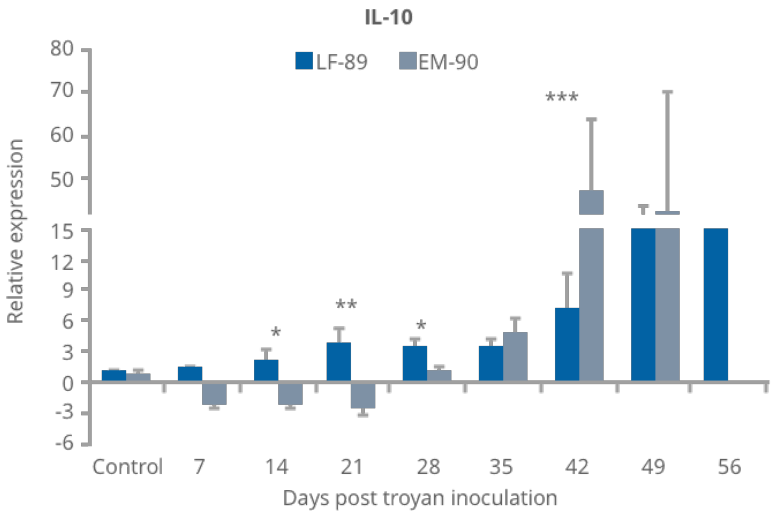

Figure 9: Relative expression of il10 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

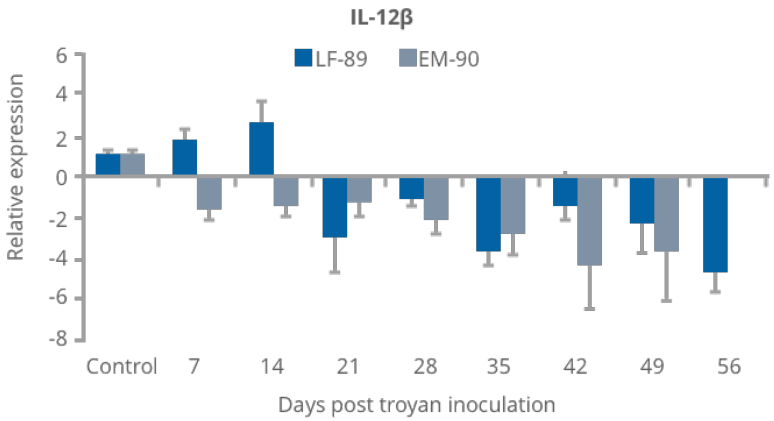

Figure 11: Relative expression of il12b in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

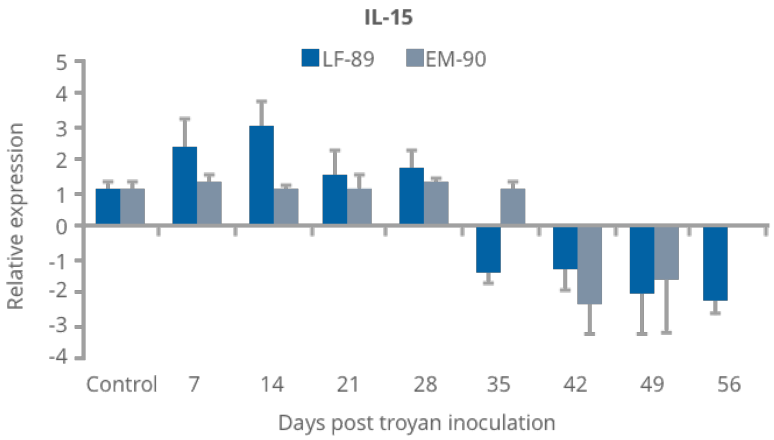

Figure 13: Relative expression of il15 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish.

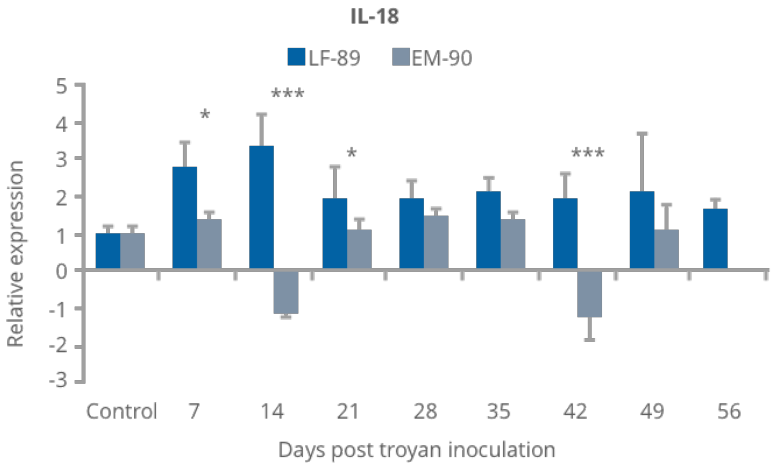

Figure 15: Relative expression of il18 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

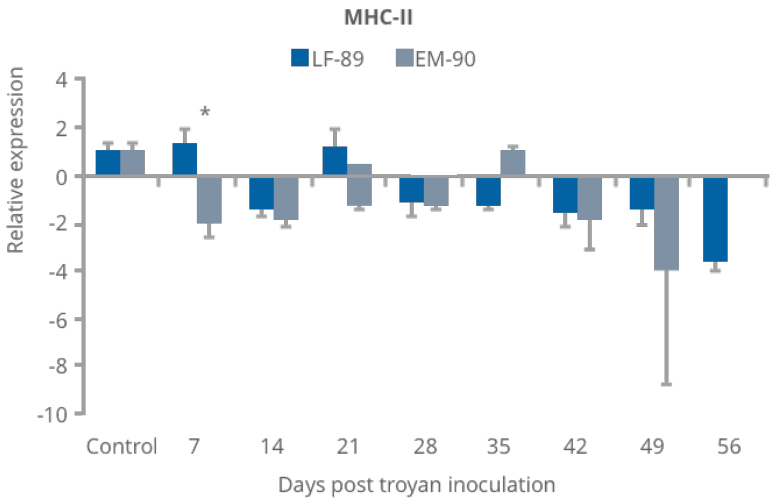

Figure 17: Relative expression of mhc2 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05

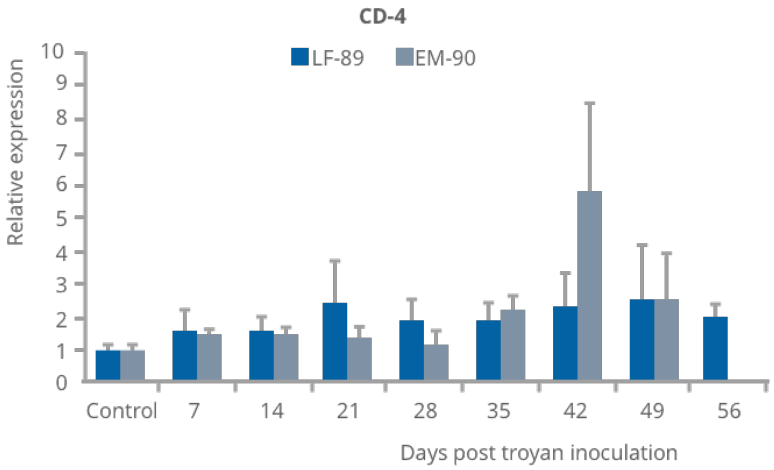

Figure 19: Relative expression of cd4 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish.

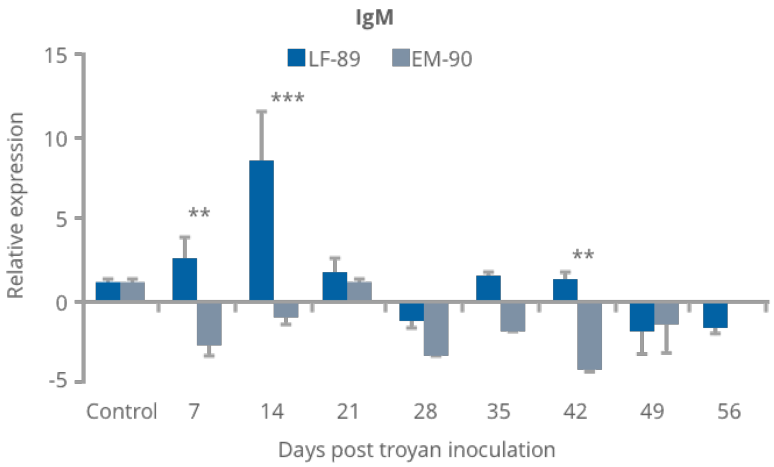

Figure 21: Relative expression of igm in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

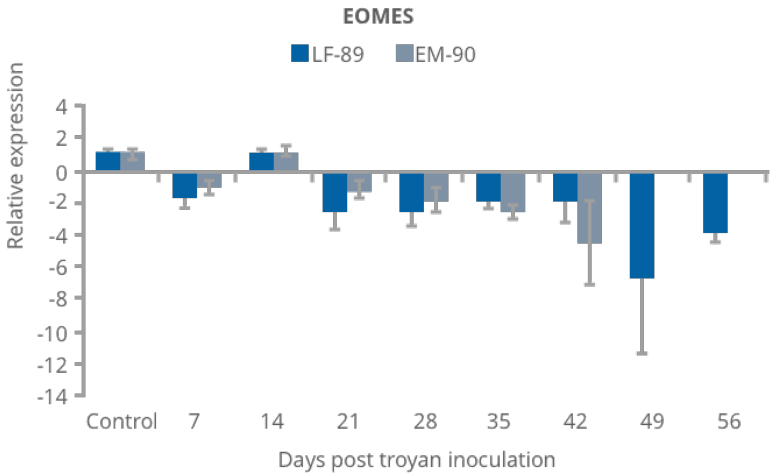

Figure 23: Relative expression of mhc2 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

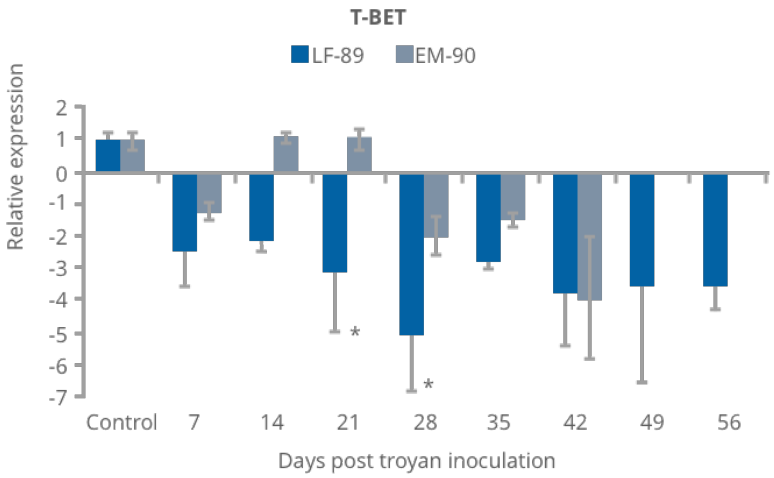

Figure 17: Relative expression of tbx21 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05.

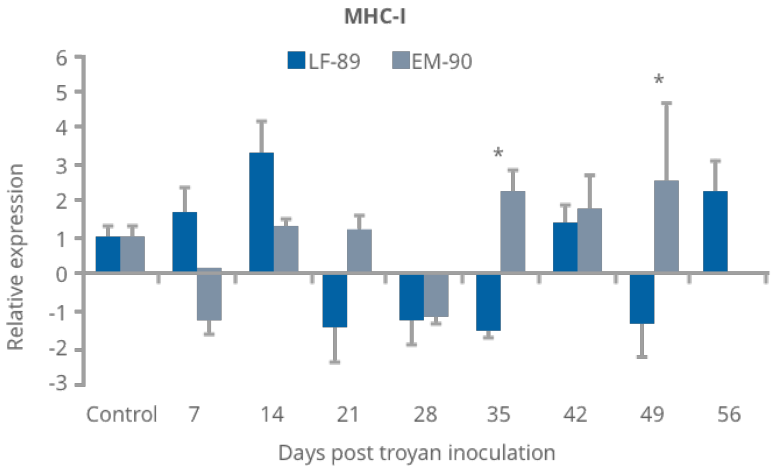

Figure 27: Relative expression of mhc1 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05.

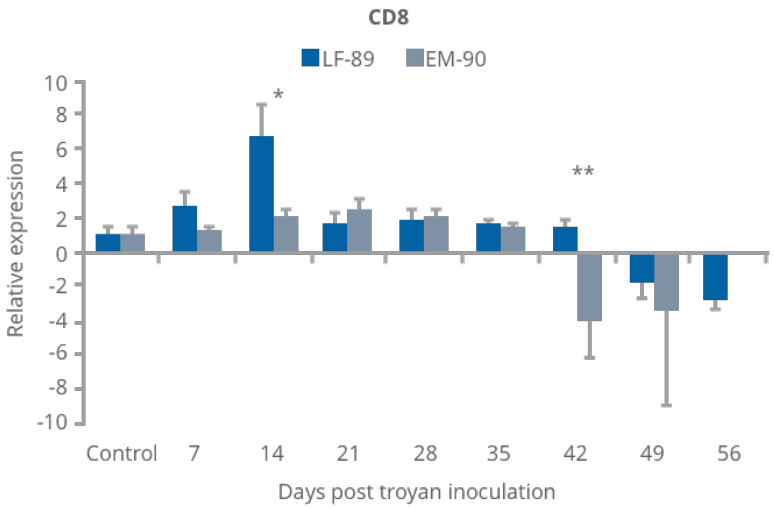

Figure 29: Relative expression of cd8b in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05 ; ***p<0,0005.

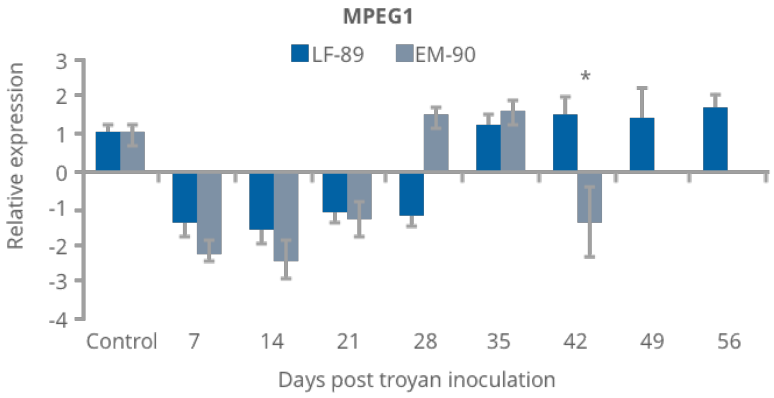

Figure 33: Relative expression of mpeg1 in anterior kidney of cohabitant fishes infected with PS-LF-89 and PS-EM-90. Each point represent the mean expression level of five fish and is described as double change +/- standard error of the mean compared with the average expression level of five control fish. *p<0,05.

Transcriptomic profile of the immune response in Atlantic salmon challenged with LF-89 and EM-90 P. salmonis isolates by means of RNA-Seq.

The results of the process are 164, 298, 60 and 170 clean transcripts for PS-LF-89 Trojans, PS-EM-90 Trojans, PS-LF-89 cohabitants, and PS-EM-90 cohabitants, respectively.

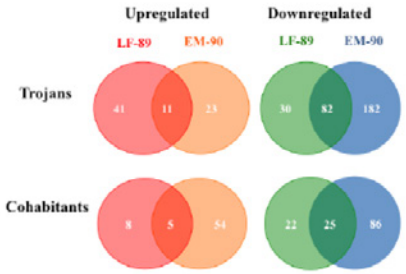

Figure 1. Venn diagram showing the number of genes trascriptionally over and under regulated through RNA-Seq in anterior kidney in troyan and cohabitant fish with PS-LF-89 and PS-EM-90. The expression of all the genes was significatelly altered ($p \leq 0.1$) with > 1 or < 1 of expression.

The Trojan and cohabitant fish infected with PS-LF-89 and PS-EM-90 presented similar patterns for biological processes (BP), molecular functions (MF) and cellular components (CC), but PS-LF-89 fish presented a larger proportion of altered genes than PS-EM-90 fish.

The altered MF were ion binding, protein binding and organic cyclic compound binding, whereas the main over-regulated and under-regulated CC were the cellular, intercellular and intracellular parts.

There is a difference in the molecular pattern for genes expressed in the anterior kidney, discovered through RNA-seq, depending on the condition of both Trojan and cohabitant fish as well as on the strain with which they were infected, PS-LF-89 and PS-EM-90, associated with immune responses and with the regulation of cellular processes. Generally speaking, the results obtained from RNA-Seq were confirmed through RT-qPCR, indicating the reliability and accuracy of the RNA-Seq expression analysis.

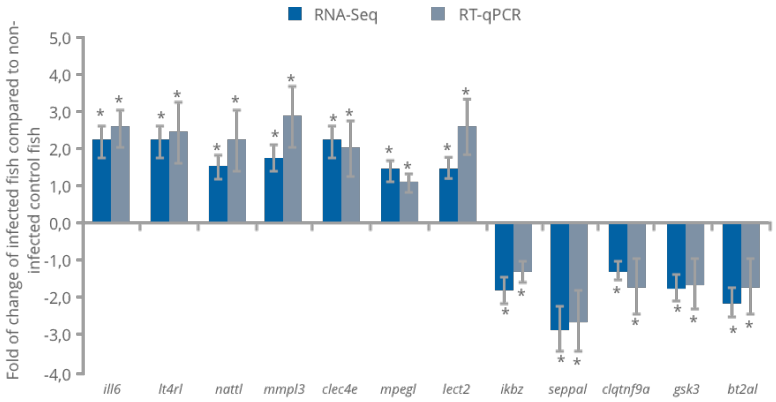

Figure 3. Quantitative confirmation through RT-qPCR of the diferential expression in anterior kidney of Atlantic Salmon challenged with the isolates PS-LF-89 and PS-EM-90, independent of the infection channel, compared to non infected control fish for 8 genes diferentially expressed previosly identified by RNA-Seq analysis. The selected transcripts were: Protein 9A related to complement 1q and tumoral necrosis factor (c1qtnf9). Protein binded to GSK-3 (gbp), z inhibitor of kappa B nuclear factor (nfkbiz), seleneprotein P (sepp1), lectine domain type C family 4 member E (clec4e), pro-interleukin 16 (il16), leucotrien receptor B4 (Itb4r) and protein similar to natterine (nattl). The bars represent the average +/- standard error of 5 fish; * significative differences ($p < 0.05$) among infected and non infected fish; + significative differences ($p<0.05$) among challenged fish PS-LF-89 and PS-EM-90

Immune response profiles at transcriptomic level that include innate, acquired and stress immunity, associated with the Salmo salar co-infection with PRV-ASCV (Piscine OrthoReovirus-Atlantic Salmon Calicivirus), PRV-IPNV (Infectious Pancreatic Necrosis Virus) and PRV-P. salmonis.

The highest percentages of positivity in healthy fish obtained from random sampling corresponded to PRV and ASCL, indicating a lower positivity for IPNV. Regarding the immune response expression patterns, differences in the non-infected fish and those having a unique positive diagnostic or a coinfection were observed.

Table 7.1. Percentage of Atlantic Salmons positive to PRV, IPNV and/or ASCV analized in the co-infection study.

	Total analized samples PRV	Total positive samples PRV
Number	312	150
%	100%	48%

	Total analized samples IPNV	Total positive samples IPNV
Number	309	8
%	100%	3%

	Total analized samples ASCV	Total positive samples ASCV
Number	277	66
%	100%	24%

Table 2. Percentage of Atlantic Salmons samples positive to more than one viral pathogen analized.

	Samples Analized by PRV+ASCV	Total positive samples PRV+ASCV
Number	275	33
%	100%	12%

	Samples Analized by PRV+IPNV	Total positive samples PRV+ IPNV
Number	274	2
%	100%	0,73%

	Samples Analized by PRV+IPNV+ASCV	Total positive samples PRV+IPNV+ASCV
Number	275	1
%	100%	0,36%

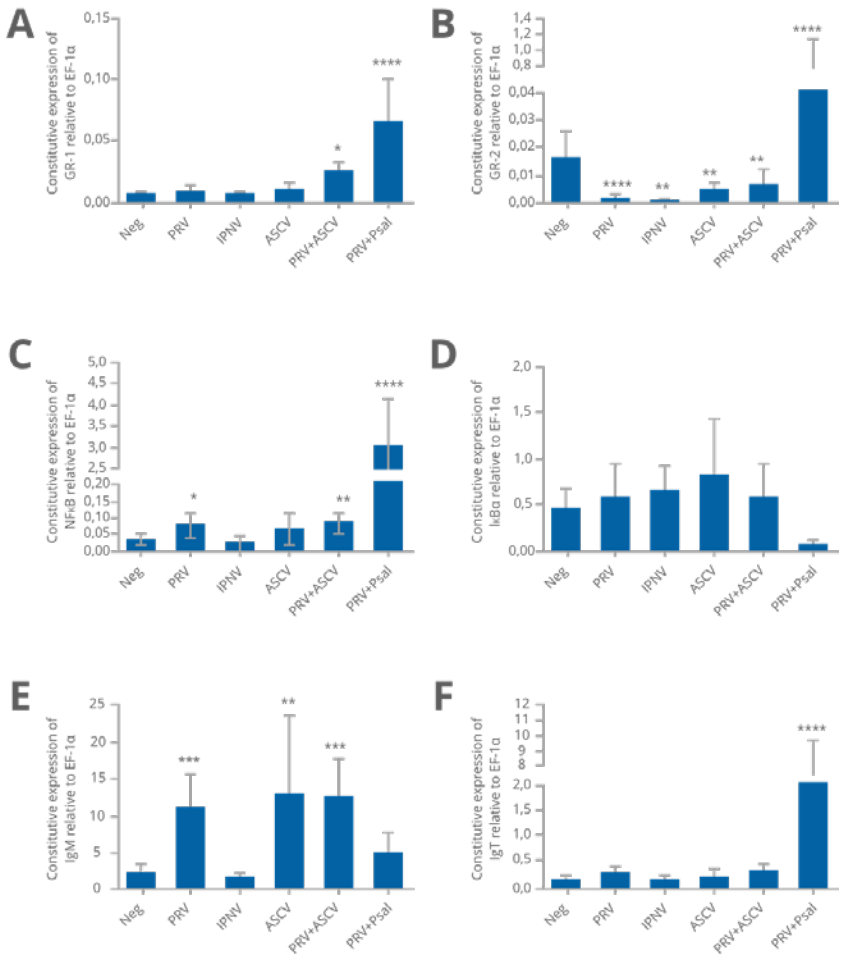

Figure 7.1. Stress and acquired immunity markers expression of (A) GR-1, (B) GR-2, (C) NFKB, (D) lKBα, (E) IgM and (F) IgT through RT-qPCR by duplicate using the expression of EF-1α as normalizer gene. A one-way ANOVA was carry out ($p<0,05$) followed by a Dunnett test (*$p<0,05$), **$p<0,01$, ****$p<0,0001$).

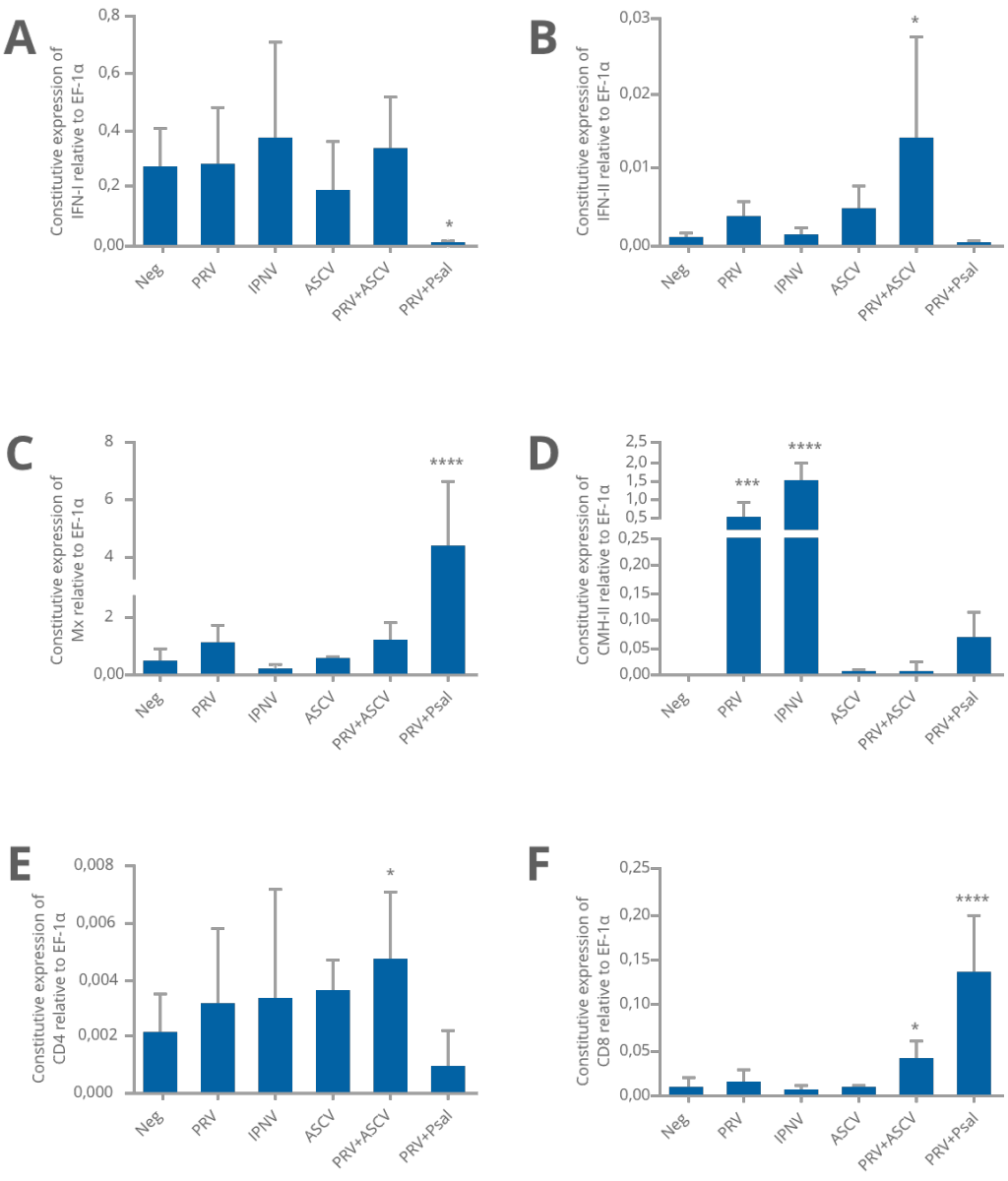

Figure 7.2. Immunity markers expression in relation to the detected pathogen and co-infection. The expression of (A) IFN-I, (B) IFN-II, (C) Mx, (D) CMH-II, (E) CD4 and (F) CD8 was analized through RT-qPCR by duplicate using the expression of EF-1α as normalizer gen. One-way ANOVA was carry out ($p<0,05$) followed by a Dunnett test (*$p<0.05$, ***$p<0.001$, ****$p<0.0001$).

DISCUSSION

The INFγ is produced by NKC and T lymphocytes in order to regulate the immune response, encouraging Th1 polarization for the antibacterial response, together with IL-12 , IL-15 and IL-18.

The TNFα is important for the antimicrobial response, including cellular proliferation and differentiation, necrosis, apoptosis and inducement of other cytokines, inhibiting the pathogen's intracellular replication. The overexpression of the tnfa in the anterior kidney of Atlantic salmon infected with both P. salmonis isolates coincides with an increase in the tnfa expression observed in the anterior kidney of Atlantic salmon infected with P. salmonis through i.p.

The IL-1β participates in the regulation of genes associated with the activation of lymphocytes, the migration of leukocytes, phagocytosis and bactericide activity. An over-expression of il1b in the anterior kidney of Atlantic salmon infected with both P. salmonis isolates was observed.

The IL-8 is involved in the early response to pathogens, inducing neutrophil, T lymphocyte and basophil migration. The over-expression of the il8 in the anterior kidney in Atlantic salmon infected with both P. salmonis isolates was observed.

The IL-12 participates in the regulation of IFNγ secretion from lymphocytes at rest, NKC stimulation and CTL maturation. The IL-10/IL-12 balance maintained by the innate immune system cells determines the Th1 polarization, which is fundamental for the control of intracellular pathogens. Fish infected with P. salmonis in the present study, regardless of the isolate, showed an over-expression of infg as well as a significant under-expression of il12b. Together, an under-expression of other genes of the humoral adaptive response (igm, mhc2) and the cellular response (mhc1, cd8b, il15, il18, eomes) was registered, indicating that P. salmonis regulates the evasion of the adaptive immune response, facilitating its intracellular survival. The IL-15 regulates the innate immune response and maintains the T cell memory; in this case, an under-regulation of the il15 and then of 35 and 42 dpi in fish infected with PS-EM-90 and PS-LF-89 was observed respectively.

The IL-18 is mainly produced by activated macrophages and it has various functions in innate and acquired immunity. An imbalance in the expression of il18, il12 and il15 alters the maturation of T cells and NKC. In this study, a slight over-expression of il18 and an under-expression of il12b and il15 are observed for both tests. The IL-10 is an anti-inflammatory cytokine mainly produced by activated monocytes and T cells as a peripheral tolerance factor and a suppressant of the immune response. The significant over-expression of the il10 in the anterior kidney in Atlantic salmon infected with both P. salmonis isolates coincides with the IL-10 inducement, described as a virulence mechanism for evading the cellular immune response and facilitating intracellular replication. At the same time, IL-10 is a potent inhibitor for IL-12. An under-expression of mhc2 induced by P. salmonis is also observed in the present study.

The isolate with the highest virulence (PS-EM-90) provoked a high over-expression of infg and a more pronounced imbalance between the over-expression of IL-10 and under-expression of IL-12 than that observed in fish infected with the less virulent isolate (PS-LF-89). An over-expression of mhc1 and an under-expression of mhc2 were observed in the present study, which coincides with regulation mechanisms of intracellular pathogens, used to produce chronic infections in the host's organism.

The under-expression of cd8 in the anterior kidney of Atlantic salmon infected with PS-LF-89 and PS-EM-90 coincides with the reduced expression of cd8 in the anterior kidney in Atlantic salmon infected intraperitoneally with P. salmonis. The generation of an efficient response of antigen-specific T cells CD8+ in order to protect against the dominant epitopes of intracellular bacteria and viruses is key for the elimination of these pathogens. Eomes is a central regulator of the development of the immunity mediated by cells, since it controls the cytolytic activity of T CD8+ lymphocytes. T-bet is a transcription factor specific to Th1 cells that controls the infg expression in Th1 cells and NKC. The reduced expression of eomes and tbx21 observed in the cohabitant fish, infected with both P. salmonis isolates confirms the bacteria's strategy of evading the adaptive cellular immune response, determining an under-expression of cd8 despite the over-expression of infg.

The isolates with a high virulence could affect the understanding of the adaptive immune response in fish infected with P. salmonis. An effective vaccine against this pathogen requires an effective activation of cellular immunity.

The results show that both P. salmonis isolates induce the innate immune response in the infected fish, even though this response is significantly heightened in fish infected with the PS-EM-90 isolate of higher pathogenicity. However, both isolates seem to inhibit the adaptive humoral and cellular immune responses. The exacerbated activation of the inflammatory response seems to increase the susceptibility of the fish, ending in a progression of the response from acute to chronic, determining an increase in intracellular replication for P. salmonis expressed by high bacterial loads, tissue damage and low survival rates, especially in fish infected with the PS-EM-90 isolate.

Understanding the cell-signaling pathways and cellular components regulated by P. salmonis during pathogenesis and the immune response allows for the improvement of SRS prevention and control alternatives. In this study, differences were observed at inflammatory mediator level, effectors: complement, lectins, antimicrobial peptides, acute phase proteins, proteases and inhibitors, extracellular matrix and cell adhesion, oxidative stress response and adaptive immunity.

At the same time, the analysis showed differences in the regulation of cell processes such as the cytoskeleton, cellular interaction and signal transduction, which is probably related to the fundamental role of the cytoskeleton in detecting intracellular bacteria and in implementing an antibacterial response, which is essential for the presentation of antigens, T cell signaling and the adaptive immune response. Other affected processes are lysosome, phagosome and endosome formations as well as the autophagy process. Fish infected with P. salmonis in the present study presented and over-expression of cathepsin Z, endosome and lysosome carboxypeptidase, participating in the migration and adhesion for T cells CD4, NKC and DCs.

Pathways such as the proliferation, progression of the cellular cycle and apoptosis: the present study and the data generated support the theory that P. salmonis induces an intensified activation of the innate immune response and of the inflammatory response, particularly in PS-EM-90 fish, but also an inhibition of the antioxidant response and the

adaptive humoral and cellular immune responses. These results seem to show a failure in the resolution of inflammation phase and the restoration of homeostasis, producing a chronic inflammation and increasing the fish's susceptibility. At the same time, both P. salmonis isolates induce a significant reorganization of the cellular cytoskeleton and an alteration of the autonomous cellular immunity, increasing phagocytosis but reducing the activity of lysosomal proteases and the degradation of proteins associated with cellular stress and ER, delays in protein transport and in antigen processing as well as delays in vesicle trafficking and autophagy. The transcriptional response induced by both P. salmonis isolates encourages the progression of the cellular cycle, of cell survival and proliferation as well as of the reduction of apoptosis. Regardless of the isolate, these mechanisms are the bacteria's strategy for evading the immune response, maintaining the viability of the cells in the host and for increasing intracellular replication and persistence in the infection site.

CONCLUSION

In accordance with the experimental conditions of the present study, we can conclude that:

- Both P. salmonis isolates induce an innate immune response, even though the response is significantly heightened in fish infected with PS-EM-90.
- Both P. salmonis isolates seem to inhibit the adaptive humoral and cellular immune responses.
- The exacerbated activation of the inflammatory response seems to increase the fish's susceptibility, ending in the progression of the response from acute to chronic, determining an increase in intracellular replication of P. salmonis, expressed in high bacterial loads, severe tissue damage and a low survival rate, especially in the group of fish infected with PS-EM-90.
- The immune response profiles at transcription level for both innate immunity and stress immunity genes in fish infected with P. salmonis are related to a transcriptional profile associated with a response to stress reflected in the increase of GR1 mRNA and the decrease of GR2 mRNA.
- Even though the inflammatory response mediated by NFB is present, it is contained by the increase of IBα expression, which is associated with an anti-inflammatory type response, related with the decrease of the IL-12 transcript during the course of the infection and the low expression of other cytokines such as IL-1, IL-8 and TNF during the first two weeks of infection. However, the increase of the expression of these cytokines, together with CD8 at the end of the infection period, suggests that these cytokines are important for the survival of fish infected with the bacteria.
- Lastly, the analysis of the profile of the IgT and IgM immunoglobulin expression suggests that the acquired humoral immune response against the bacteria is mainly associated with IgT expression in the anterior kidney in S. salar. However, it is necessary to establish

the levels of these immunoglobulins at blood protein level, as well as their specificity and to determine the functional effect of the P. salmonis infection on these immunoglobulins.

- In accordance with the obtained results, no classic differential expression profiles are discerned for the genes GR1, GR2, NFB or IB, associated with a response to stress in the specimens analyzed in this study.
- In addition, we can conclude that viral infections and co-infections present an increase in the IgM expression. Regarding IgT, its expression is connected to the presence of P. salmonis in co-infections with PRV.
- Moreover, there is a significant inducement of the CD8 immunity cellular markers in fish co-infected with PRV-ASCV and PRV-P. salmonis, related to the regulation of lymphocytes T CD8+, associated with cytotoxic activity against intracellular pathogens.
- Furthermore, the inducement of IFN-II in the group of co-infected fish suggests its connection with NK cell and CD8+ T cell cytotoxic activity, associated with the elimination of intracellular pathogens in fish.


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