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Antiviral effects of Alpinone on Atlantic salmon (*Salmo salar*) infected with the infectious pancreatic necrosis virus

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ABSTRACT

Infectious pancreatic necrosis virus (IPNV) poses a significant threat to the aquaculture industry, primarily affecting juvenile salmonids, which often experience high mortality rates. Several therapies are being developed to control infections in fish, such as vaccines and immunostimulants; however, antiviral therapies are scarce and have limited effectiveness. Therefore, the search for compounds that can enhance antiviral responses is crucial for the sustainable growth of aquaculture. This study aimed to contribute to the search for new therapeutic agents to control Infectious Pancreatic Necrosis Virus (IPNV) by evaluating the antiviral effects of Alpinone in an experimental infection in Atlantic salmon (*Salmo salar*). A total of ninety fish, each weighing approximately 15 grams, were randomly selected and divided into three groups. The fish were intramuscularly injected with one of the following treatments: (i) 50 μg of Alpinone in saline (n=30), (ii) a 3% Montanide solution (n=30), or (iii) a saline solution (n=30). On day 30, all fish received an intraperitoneal injection of the IPNV (VR-299 strain). On day 30, all fish received an intraperitoneal injection of IPNV (VR-299 strain). After euthanizing the fish, head kidneys and gills, the latter a mucosal tissue that in natural infections serves as the initial point of contact with microorganisms, were collected for analysis on days 0, 5, and 26 post-infection. Signs associated with the infection were recorded on day 5 post-infection. The analysis showed that Atlantic salmon treated with Alpinone 30 days before IPNV infection exhibited fewer clinical signs of the disease and a reduction in viral load by three orders of magnitude compared to both Montanide-treated and untreated fish. The gene expression of immune response indicators, including *ifn-γ, il-8, il-12β, tgf-β*, and *mx*, was analyzed in the gills using real-time PCR. The results showed that the expression of *il-8* and *il-12β* increased on day 5 after IPNV infection solely in the Alpinone-treated fish, which may be related to the lower viral loads and fewer clinical signs present in this group. Overall, this study demonstrated that Alpinone has a significant antiviral effect on IPNV infection in Atlantic salmon, which correlates with elevated levels of genes encoding IL-8 and IL-12, two pro-inflammatory and immunomodulatory cytokines that enhance the host's defense mechanisms in fish.

Introduction

The infectious pancreatic necrosis virus (IPNV), which belongs to the Birnaviridae family, causes a deadly and highly contagious disease known as infectious pancreatic necrosis, affecting several fish species, including Atlantic salmon [1]. This virus is one of the four leading causes of mortality in marine aquaculture, accounting for 13,4 % of deaths attributed to infectious agents in rainbow trout (*Oncorhynchus mykiss)* and Coho salmon *(Oncorhynchus kisutch)*, however, exhibits the highest mortality rate associated with this virus [2]. The impact of IPNV infection on juvenile fish is particularly severe, with the highest mortality occurring between days 4 and 5 post-infection [3]. In addition to signs of anorexia, including weight loss and decreased food intake, other concerning symptoms include abdominal distension and irregular swimming behavior. One aspect to consider about this virus is the presence of various strains that arise from mutations; these can be regarded as part

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of the ability of RNA viruses to adapt to the environment. This variation has resulted in identifying several virulence factors associated with the virus structure, the most significant being the VP2 protein. This protein is part of the virus capsid and is critical in virus recognition [4].

Several therapies targeting IPNV aim to enhance the fish immune response through vaccines and immunostimulants or directly target and inhibit IPNV replication. Various vaccines have been developed using recombinant proteins, DNA, or viral-like particles (VLP) [5–7]. Although these vaccines induce responses, including production of neutralizing antibodies [8], they require improvements to produce protection and eliminate viruses [9]. In this context, identifying more immunogenic antigens [10] and discovering effective immunostimulants for potential inclusion in vaccine formulations is an important research task in the field. Considering compounds that can directly inhibit the virus, there are no commercially available treatments for this virus in aquaculture. Finding effective agents to combat IPNV has proven to be quite challenging.

A global issue for immune therapies is the scarcity of adjuvants and immunostimulants that can be added to vaccines to protect species from potential viral infections effectively [11], as well as the scarcity of antiviral agents that directly interfere with virus replication. Therefore, there is increased interest in exploring more natural compounds to find those able to enhance antiviral responses while minimizing toxicity compared to conventional treatments [12]. In fish, recent studies have demonstrated that compounds such as andrographolide, fucoidan, and lambda-carrageenan can reduce the viral load of IPNV in infected cells, even after infection occurs [13,14]. This indicates that these compounds, recognized for their immunostimulant properties, can help establish an antiviral state against IPNV. Latest research in humans and animal health has identified flavonoids as a promising bioactive compound. Flavonoids are a type of natural compound characterized by diverse phenolic structures. These compounds exhibit various biological activities, including antioxidant, antiviral, antiallergic, anticancer, and anti-inflammatory properties [15]. In this study, we aim to identify a novel therapeutic agent against fish IPNV infection by testing Alpinone, a flavonoid derived from the resinous exudate of *Heliotropium huascoense* [16]. Alpinone plays a regulatory role in the fish immune response by increasing the transcriptional expression of the genes encoding RIG-I and MDA5 cytosolic receptors, as well as antiviral molecules like Interferon (IFN)-a and Myxovirus resistance protein (Mx) in the kidney cells of Atlantic salmon [17]. Additionally, Alpinone enhanced in vivo expression of *tumor necrosis factor (tnf)-α, interleukin (il)-1, interferon (ifn)a, ifn-transforming growth factor (tgf)-β1* in the kidney of Atlantic salmon and shows in vitro antiviral activity against Infectious Salmon Anaemia virus [18]. These findings support the use of Alpinone as an immunostimulatory and potential antiviral agent against different viruses in fish. Therefore, here we specifically assessed the antiviral effects of Alpinone against experimental infection of Atlantic salmon (*Salmo salar*) with IPNV.

Material and methods

Ethics statement

Fish management adhered to the regulations set by the Ethics Committee of the University of Santiago de Chile and followed the guidelines of the funded research project. The Institutional Ethics Committee reviewed the Fondecyt Project No. 11170984, considering the principles of the Three Rs [19] and Chilean Law 20,380 on the protection of animals. This law, specifically Title IV, pertains to experiments involving live animals and covers Articles 6, 7, 8, 9, and 10.

Propagation of IPN virus in CHSE-214 cells

Briefly, Chinook salmon embryo (CHSE-214) cells (ATCC number CRL-1681) were used for the propagation and titration of the IPNV (VR-299 strain, generously donated by Dr. Sandino), following the protocol by Reyes-López [20]. The lysis plate method for IPNV was employed to determine the viral titer [21].

Alpinone isolation, purification and characterization

The extraction of Alpinone was conducted following the method previously outlined [18]. In summary, samples of *Heliotropium huascoense* Johnston were collected during the flowering season in the III region of Chile. The resinous exudates were extracted by immersing 900 g of the entire plant in dichloromethane (CH_2Cl_2), after which the extract was concentrated. The compound (2S, 3S)-3,5-dihydroxy-7-methoxyflavanone ((−)-Alpinone) was purified according to the procedure described [16]. Nuclear Magnetic Resonance (NMR) spectroscopy confirmed that the purity of Alpinone exceeded 97 %. The purified Alpinone was then dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.5 % (v/v), and the stock solution was stored at 4°C.

Fish treatments

Atlantic salmon were sourced from a local fish farm and maintained in tanks with a freshwater system at a biomass of 10–12 kg/m^3, with a temperature range of 12–16°C and continuous aeration. The fish were fed with commercial pellets daily and allowed to acclimate for one week before treatment. Approximately 15 g fish were randomly selected and divided into three groups. Each fish in the experimental group was intramuscularly injected with 50 μg of Alpinone dissolved in a sterile 0.9 % NaCl solution (n=30). The control groups received either an injection of 50 μL of sterile 0.9 % NaCl solution (n=30) or a 3 % Montanide ISA 763A VG solution (n=30). On day 30, all fish were intraperitoneally injected with 50 μL of an IPNV stock, which had an infectious titer of 10^7 $TCID_{50}$/mL. The titration was performed using lysis plaques in CHSE-214 cells. Ten fish from each group were euthanized with an overdose of benzocaine at day 0 and on days 5 and 26 post-infection (dpi). Head kidneys and gills were extracted from each specimen and stored at -80°C for analysis. Signs associated with the infection were recorded on day 5 post-infection.

RNA extraction and DNase treatment

For RNA extraction, tissue from each gill or head kidney tissue was homogenized in 1 mL of RNA-Solv (Omega®). Next, 200 μL of chloroform was added, and the mixture was vortexed using a DLAB® vortex mixer. The mixture was centrifuged (Digicen 21TM, Ortoalresa®) for 15 min at 9000 x g and 4°C. Following centrifugation, 500 μL of the aqueous phase was gently mixed with 500 μL of cold isopropanol and incubated at -20°C for 30 min. After incubation, the sample was centrifuged for 10 mins at 9000 x g and 4°C. The supernatant was removed, and 600 μL of 75 % ethanol was added before centrifuging for 5 min at 9000 x g and 4°C. The supernatant was discarded, and the total RNA was resuspended in nuclease-free water and stored at -80°C. For DNase treatment, 5 μg of RNA was combined with 1 μL of 10X Buffer (Promega®), 1 μL of DNase (Promega®), and nuclease-free water, following the manufacturer's instructions. This mixture was incubated for 30 min at 37°C. After incubation, 1 μL of EDTA (25 mM) was added, and the mixture was incubated at 65°C for an additional 10 min.

RT-PCR

To obtain cDNA, 11 μL of RNA were mixed with 0.5 μL of dNTP (10 mM) and 1 μL of Oligo(dT) (0.5 μg/μL) from Promega®. Next, we added 5 μL of 5X buffer (Promega®) and 6.5 μL of nuclease-free water. Then, 1 μL of MMLV enzyme (200 U/μL) (Promega®) was incorporated and the mixture was incubated at 42°C for 60 min. Finally, the reaction was terminated by incubating the mixture at 70°C for 15 min. For real-time PCR was performed with a SensiMix SYBR® Hi-ROX kit (Bioline), 2 μL of

the cDNA, 1.25 μL of each primer (10 μM), 12.5 μL of 2X buffer (Bio-Line®), and 8 μL of nuclease-free water were mixed. The mixture was first incubated at 95°C for 5 min. Then, 40 PCR cycles using the Exicycler ™ 96 (Bioneer®) were performed with the following thermal profile: 95°C for 5 min, followed by 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. The reference gene for gene expression analysis was the elongation factor 1α (*ef1a*) [22]. The expression levels for each tested gene are shown as normalized relative expression (NRE) [23]. The mathematical representation of the equation is:

$$\mathrm{NRE} = \frac{E\text{^}\mathrm{dCT\ target\ gene\ (control - treated)}}{E\text{^}\mathrm{dCT\ reference\ gene\ (control - treated)}}$$

Viral load quantification

Quantification was conducted using an equal amount of tissue from each specimen through reverse transcription quantitative PCR (RT-qPCR) targeting the VP2 gene (Table 1). The IPNV RNA copies were calculated from the calibration curve created using RNA standards, which correlates with the Cq values obtained from RT-qPCR of the VP2 gene. Real-time PCR reactions were performed in 96-well plates (AXI-GEN®) utilizing the Stratagene Mx 3000P equipment (Agilent Technologies®). The PCR mixture for quantification included the following components: 0.8 μL of Forward Primer (10 μM); 0.8 μL of Reverse Primer (10 μM); 10 μL of 2x SensiFast SYBR Hi-Rox (BioLine®); 0.2 μL of Reverse Transcriptase (BioLine®); 0.4 μL of RiboSafe RNase inhibitor (BioLine®); 4 μL of warm buffer; and 3.8 μL of free-nuclease H_2O. The thermal cycling profile was as follows: 1 cycle at 45°C for 10 min, one cycle at 95°C for 2 min, 40 cycles at 95°C for 5 s, 60°C for 20 s, and 72°C for 5 s. The quality of the PCR products was monitored using post-PCR melt curve analysis. Data was analyzed using MxPro QPCR software (Agilent Technologies, USA).

Statistical analysis

GraphPad Prism 10.5.0 for MacOSX was used for statistical procedures and graph drawing. Statistical analysis for viral load was conducted using one-way ANOVA followed by Tukey's post hoc, while gene expression evaluation after treatment and over time was performed using two-way ANOVA with Tukey's multiple comparisons test. $p < .05$ was considered statistically significant.

Results

Effects of Alpinone on infectious pancreatic necrosis signs and viral load

The effects of Alpinone on Atlantic salmon IPNV infection were evaluated by measuring the disease signs and the viral load in the kidneys of the infected salmon, as the kidney has been described as one of the organs where primary replication of IPNV occurs [24]. Three groups of fish were injected with different treatments: one group received Alpinone, another received Montanide as a control, and the last group served as the negative control, being injected with saline. No abnormal behavior or disease signs were observed after the administration of these substances. However, following a challenge with the virus, three signs commonly associated with Infectious Pancreatic Necrosis Virus (IPNV) infection, i.e., bulging abdomen, exophthalmia, and anorexia, were observed around day 5 post-infection. The highest number of fish exhibiting these signs was found in the control and Montanide groups (Fig. 1).

In contrast, the group treated with Alpinone showed a significantly lower incidence of these signs (Fig. 1). No deaths were observed in any of the groups following the infection. The viral load in the kidneys of both treated and control fish was measured after infection by quantifying the copies of the VP2 gene. On day 5 post-infection, fish of the three groups exhibited the same viral load (Fig. 2), while on day 26, the control group and the Montanide-treated group exhibited approximately the same viral load, with values of 0.7×10^6 and 0.85×10^6 copies of the VP2 gene, respectively (Fig. 2). In contrast, the group treated with

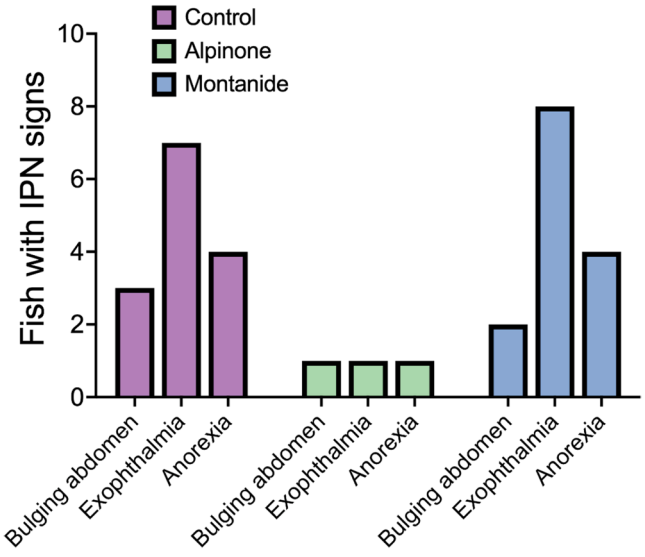

Fig. 1. Clinical signs of IPN at the peak of infection for each treatment. On day 5 post- infection, bulging of the abdomen, exophthalmia, and anorexia were identified as signs of infection when the highest mortality rate occurred. Each bar represents the number of fish showing the corresponding sign. Some fish showed more than one clinical sign.

Table 1
Real time PCR primer sequences.

Gene Name	Sequence (5- 3)	Eff	Size (pb)	Accession number
IFN-γ	Fw: CCGTACACCGATTGAGGACT	2.08	133	NM_0011235
Interferon gamma	Rv: GCGGCATTACTCCATCCTAA			
TGF-β	Fw: AGCTCTCGGAAGAAACGACA	2.07	136	EU082211
Transforming growth factor beta	Rv: AGTAGCCAGTGGGTTCATGG			
IL-8	Fw: GCCCTCCTGACCATTACTGA	2.01	186	BT046706
Interleukin-8	Rv: AAATCTCCTGACCGCTGTTG			
IL-12 β	Fw: GAGCCAAGTCTTATGGCTGC	2,08	159	XM_014205516.1
Interleukin-12 beta	Rv: GTTCAAACTCCAACCCTCCA			
Mx	Fw: TGTAACACGATGCCCTCTCG	1.98	211	NM_001139918.1
Myxovirus resistance protein	Rv: GACGTCAGGGGAGCCAATC			
EF1a	Fw: GGGTGAGTTTGAGGCTGGTA	2.09	156	NM_001141909.1
Eukaryotic translation elongation factor 1 alpha	Rv: TTCTGGATCTCCTCAAACCG			
VP2	Fw: GACCAAGTTCGACTTCCAGC			[48]
Viral protein 2	Rv: ATCGGCTTGGTGATGTTCTC			

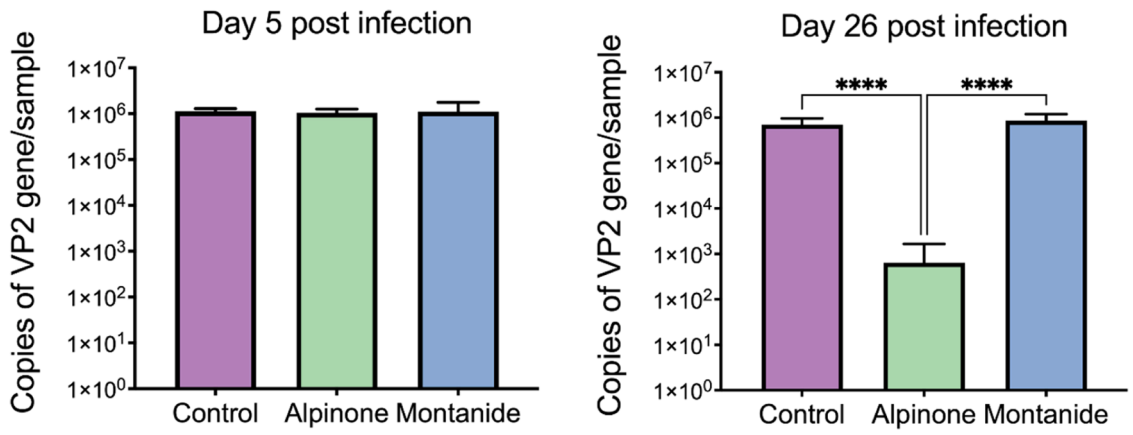

Fig. 2. Virus load in the head kidney of infected fish. Viral load was assessed by quantifying the number of VP2 gene copies in the head kidney of IPNV-infected fish. Sampling was performed on day 5 post-infection (n=42) and on day 26 post-infection (n=38). Green bars represent the group of fish treated with Alpinone. Blue bars represent the fish treated with Montanide. Violet bars are the control group. The data is shown as mean and SD and was statistically analyzed using one-way ANOVA followed by Tukey's post hoc to determine significant differences (*p<.05; ****p<.0001).

Alpinone displayed a viral load of 0.64 × 10^3 viral copies, i.e., a thousand times lower than the other groups (Fig. 2).

Gene expression of immune genes in gills after compound treatment and IPNV infection

The effect of treatments on the expression of immune genes was measured in gills because they are one of the main entry points for the virus, and the immune tissue associated with the mucosa of this organ should serve as a primary barrier of protection against IPNV [1]. On day 30 after treatment with Alpinone or Montanide, which coincided with the day of IPNV infection (referred to as day 0), there was no difference in the expression of any of the tested genes—specifically *il-8, ifn-γ, tgf-β,* and *mx* between the study groups (Fig. 3). After IPNV infection, the control group showed no changes in the expression of all the tested genes over time (Fig. 3, violet bars). In the Alpinone-treated fish, *il-8* and *il-12β* expression showed approximately twofold increase 5 days post-infection (dpi) compared to time zero. The expression then decreased to the baseline levels by 26 days post-infection (Fig. 3, green bars). There was a clear difference between the treatments, as the gene expression of both genes did not change after infection in the Montanide-treated group compared to the baseline (Fig. 3, blue bars). Regarding the expression of *ifn-γ*, there was a significant decrease after infection on days 5 and 26 post-infection in both the Alpinone and Montanide-treated groups (Fig. 3). The expression levels of *tgf-β* remained unchanged on days 5 and 26 in the Alpinone-treated group, while there was a significant upregulation on day 26 post-infection in the Montanide-treated group (Fig. 3). Finally, no changes were observed in the expression of *mx* under any of the studied conditions (Fig. 3). Overall, the administration of Alpinone to fish 30 days prior to IPNV infection resulted in a significant immune response in the gills of the infected fish, response highlights the potential effectiveness of this treatment in enhancing immune function against the virus.

Discussion

In this study, we found that pre-treating Atlantic salmon with Alpinone before exposing them to the Infectious Pancreatic Necrosis Virus (IPNV) led to lower viral loads in the kidneys and fewer clinical signs of the disease compared to untreated fish. Since Alpinone is a flavonoid, this finding aligns with research showing that flavonoids have antiviral effects against DNA and RNA viruses [25]. For example, epigallocatechin gallate has activity in vitro and in vivo against human viral infections caused by adenovirus type 2 and enterovirus 71 [26,27], while the methylated flavonol isorhamnetin shows strong antiviral effects against influenza virus [28]. Viruses that infect fish species are also targets of the antiviral activity of flavonoids. For instance, Kang et al. demonstrated that fisetin exhibits antiviral activity in vitro against both the infectious hematopoietic necrosis virus (IHNV) and the viral hemorrhagic septicemia virus (VHSV) [29], while we reported that 7-O-methyleriodictyol, as well as Alpinone, have antiviral properties against the infectious salmon anemia virus (ISAV) [18,30]

Although the development of quantitative trait loci (QTL)-dependent IPN-resistant broodstock salmon has positively impacted the salmon industry by reducing virus outbreaks [31], the emergence of new variants and reports of significant outbreaks in farmed salmon indicate that IPNV continues to pose a threat to the aquaculture industry, particularly in Chile [32,33]. This finding highlights the potential of Alpinone as a new therapeutic option to safeguard fish from IPNV exposure. While we administered the flavonoid via injection for controlled experimental conditions, the dosage and route of administration, such as oral through feed, remains an open subject for exploration.

One of the possible mechanisms responsible for the Alpinone antiviral activity may be at least partly due to its immunostimulant properties. However, Alpinone effects on gene expression observed 30 days after treatment showed no differences compared to the control group of fish. The apparent lack of response to Alpinone may be due to the timing of our measurements, which were taken after 30 days of treatment, to align with the protocols for administering functional products on farms. In this experimental approach, we anticipated that the effects of Alpinone treatment might not be observed at this stage but would become evident later, following stimulation with a pathogen or after vaccination. In fact, when fish were IPNV-infected, those previously treated with Alpinone showed an increase in *il-8* and *il-12β* in gills on day 5, which can be associated with the fewer clinical signs and reduced viral loads observed in this group. Increased expression of *il-8* and *il-12* is consistent with the fact that gills, as part of the mucosal-associated lymphoid tissues (MALTs), contain many immune cells to counteract the effects of microorganisms that may adhere and invade the fish [34].

IL-8 is an inflammatory cytokine and chemokine that plays a role in both innate and adaptive immunity. Its upregulation triggers the migration of neutrophils, T lymphocytes, and basophils to the site of infection [35]. Similarly, fish IL-8 can induce the migration of macrophages in Ayu (*Plecoglossus altivelis*), rainbow trout and large yellow croaker (*Larimichthys crocea*) [36–38]. Fish IL-8 significantly increased in the fin, gill, and spleen of olive flounder infected with VHSV and in the head kidney and spleen of sea trout after 1–2 days post-infection with IPNV, suggesting that fish IL-8 may limit the virus spreading in early days post-infection [39]. In contrast, the present study on IPNV infection of Atlantic salmon, along with previous research on persistent

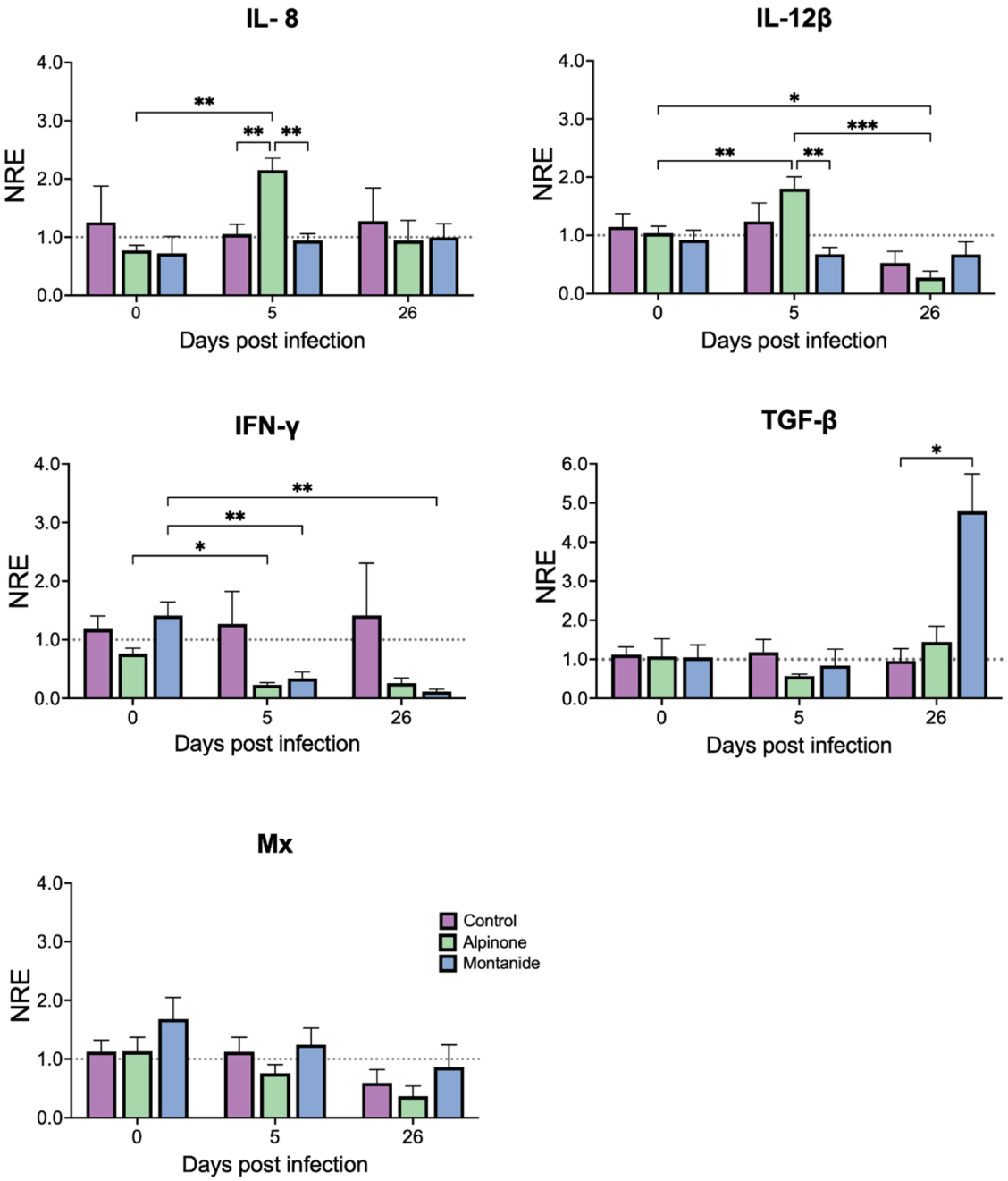

Fig. 3. Expression levels of immune genes in the gills of IPNV-infected fish. The gene expression of the cytokines was measured at the indicated time points after infection. The bars represent the groups of fish receiving treatments 30 days before infection. Green bars represent the group of fish treated with Alpinone. Blue bars represent the fish treated with Montanide. Violet bars are the control group. Each bar represents data obtained from 5 to 9 fish. The data represents Mean and SEM and were statistically analyzed by applying a two-way ANOVA with Tukey's multiple comparisons test. to determine significant differences (*p < .05; **p < .01; ***p < .001; ****p < .0001).

IPNV infection in rainbow trout, suggests that the low or absent upregulation of *il-8* can compromise the antiviral immune response [40]. Since our study showed that Alpinone, used as an immunostimulant in Atlantic salmon, facilitates the early production of *il-8* in the gills during infection, we hypothesized that this response may enhance the recruitment of leukocytes at one of the IPNV entry sites, resulting in a robust antiviral response, as we observed.

The IL-12β is one of the chains of the cytokines IL-12 and IL-23 (both IL-12 family members) which are produced by macrophages and dendritic cells in mammals. Fish macrophage-like cells have been described in some fish species [41], and they can produce IL-12, especially in response to infections [42]. In mammals, it is well known that IL-12 stimulates the production of IFN-γ by natural killer (NK) cells and T cells, particularly CD4+ T helper 1 (Th1) cells [43]. In turn, IFN-γ can further enhance the production of IL-12 from antigen-presenting cells, creating a positive feedback loop that amplifies the Th1 response. In our analysis, treatment with Alpinone resulted in the upregulation of *il-12* and downregulation of *ifn-γ* in the gills of fish five days post-infection with IPNV, suggesting a distinct type of immune regulation in Atlantic salmon that may help develop a regulated immune response associated with fish resistant to IPNV challenge [20].

Montanide, used as a vaccine adjuvant, was employed as a reference

to illustrate that Alpinone might also function as an adjuvant. Reports indicate that when injected into rainbow trout, Montanide induces increased expression of immune-related genes [44]. However, no effects on *il-8* and *il-12β* expression were observed in fish treated with the adjuvant. Montanide treatment produced downregulation of *ifn-γ* expression five days after IPNV infection. Only the fish from the Montanide group showed an increase in *tgf-β* expression in their gills at 26 days post-challenge. This finding aligns with the known ability of Montanide to promote the upregulation of foxp3 [44], as both TGF-β and foxp3 are expressed by regulatory T cells (Tregs) able to suppress immune responses during infections. The myxovirus resistance protein (Mx) plays a role in preventing the assembly of viral nucleocapsid protein (N), inhibiting the formation of new virus particles [45–47]. The expression levels of *mx* did not change in any of the three groups infected with IPNV. This indicates that Mx did not play a role in the response to the infection or in the reduction of viral load observed in the fish treated with Alpinone.

Conclusions

Altogether, this study demonstrates the antiviral effects of Alpinone against IPNV infecting Atlantic salmon. The reduction in viral load and decrease in disease signs observed in the Alpinone treated fish were not observed in the control and Montanide groups. Results are consistent with previous findings, indicating that Alpinone decreases the number of viral gene copies of infectious salmon anemia virus in vitro. Further studies are needed to understand the mechanisms of antiviral activity better and develop strategies to utilize Alpinone in fish farms as a prophylactic treatment against viral agents.

CRediT authorship contribution statement

Paz Basualto-Díaz: Writing – original draft, Methodology, Investigation, Data curation. **Almendra Benavides:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Data curation. **Daniela Gutierrez:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Nadia Epuyao:** Validation, Methodology, Data curation. **Brenda Modak:** Writing – review & editing, Resources, Funding acquisition. **Mónica Imarai:** Writing – review & editing, Visualization, Validation, Resources, Investigation, Funding acquisition, Formal analysis, Data curation. **Beatriz Valenzuela:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Competing interest statement

We have no competing interests to declare.

Data availability

Data will be made available on request.

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