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The efficacy of new oral vaccine feeds against *Salmonid novirhabdovirus* in rainbow trout

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ABSTRACT

Salmonid novirhabdovirus (IHNV) causes infectious haematopoietic necrosis (IHN) in salmonid species. Despite an injectable plasmid-based DNA vaccine of the glycoprotein (G) gene is effective, there are no oral vaccines for mass vaccination of rainbow trout (*Oncorhynchus mykiss*) fry. Recombinant baculoviruses were generated, used in cabbage looper (*Trichoplusia ni*) insect larvae to produce IHNV G and IHNV G-C5a proteins. Western blotting and chemiluminescence assays confirmed the expression of recombinant proteins, which were added to the fish feeding and top-coated with unflavored gelatin binder. Commercial rainbow trout were fed with experimental diets containing either IHNV G or IHNV G-C5a proteins for 2 weeks, and boosted 4 weeks after. Four weeks post-booster, fish were challenged with IHNV by immersion. Survival upon the infection challenge was evaluated. Spleen were sampled at 7 and 14 days post infection (dpi). Non-vaccinated and IHNV G fed trout reached a mortality of 91.7 and 97.6%, and 70.9 and 88.4%, respectively at 8 and 15 dpi. The IHNV G-C5a fed group exhibited a reduced mortality of 51.2% at 8 dpi, reaching 81.7% at 15 dpi, suggesting some level of antiviral protection. The individual viral load was measured by RT-qPCR detection of IHNV N gene, showing no significant difference across experimental groups. The transcription modulation of selected immune response markers was evaluated across experimental groups, including Type I IFN-a, Mx-1, CD4, and IgM. Further study is needed to assess how new oral vaccines may become effective to mitigate IHNV pathogenesis in juvenile trout by modulating the host immune response to protect towards IHNV exposure.

1. Introduction

Infectious haematopoietic necrosis virus (IHNV) is one of the most important viral pathogens, causing systemic and often virulent disease predominantly in both wild and cultured salmon and trout species [1,2]. The first reported epidemics of IHNV occurred in sockeye salmon (*Oncorhynchus nerka*) fry at Washington and Oregon fish hatcheries during the 1950′s [3,4]. By the mid-1970′s, IHNV was endemic in sockeye throughout Alaska, but became endemic in rainbow trout (*O. mykiss*) throughout the Hagerman Valley trout-farming region in Southern Idaho [5,6]. Since then, IHNV has spread to Asia and Europe and continues to persist at multiple sites causing epidemics, which may approach 70–80% mortality depending on the life-stage of the fish host [1,7]. IHNV isolates are grouped in three main genogroups, based on their geographical range across the Pacific Northwest of America [7,8]. IHN is currently a notifiable disease listed by the World Organisation for Animal Health (WOAH) [9], and prevalent in several countries and trading areas.

Salmonid novirhabdovirus is the type species of the genus *Novirhabdovirus*, within the family *Rhabdoviridae* [10]. It has a linear single-stranded, negative-sense RNA genome of approximately 11 kb [11]. The genome comprises of six genes, in the order 3′-N-P-M-G-NV-L-5′ [12,13]. The RNA is tightly associated with the nucleoprotein (N), phosphoprotein (P), and RNA-dependent RNA polymerase (L) to make an RNP-complex, which interacts with the matrix protein (M) to give a bullet-like structure. The glycoprotein (G) is the major host-protective antigen of the virus, which induces the neutralizing antibodies in fish [14]. Therefore, the G gene has been a major target towards the development of vaccines against IHNV [15,16].

Since 1989, many researchers have tried to develop effective and safe vaccines by producing the recombinant G protein in bacterial and eukaryotic systems, or the G gene plasmid DNA as potential vaccine

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candidates to control IHN pathogenesis [17–20]. Engelking and Leong used purified G protein (produced in bacteria) to immunize fish by immersion (~50 mg/mL) and demonstrated that it can provide substantial protection to rainbow trout and sockeye salmon upon IHNV challenge [17]. In another study, Cain and coworkers demonstrated that fish immunized by IP injection with the recombinant IHNV G protein (produced in *Spodoptera frugiperda* [Sf-9] insect cells using a baculovirus) provided very limited protection in rainbow trout challenged with IHNV [18]. In the same year, Kurath's group reported that plasmid-based DNA vaccine of the G gene administered by intramuscular injection elicited complete protection upon IHNV challenge [19]. Although this vaccine is very effective, it is not practical for fry, and it is labor-intensive. To overcome this issue, Ballesteros *et al.* reported that an oral DNA vaccine against IHNV, encapsulated in alginate microspheres, can give moderate protection in rainbow trout after administration of a high dose of plasmid-based G gene (100 μg) [20]. This dose is 20 folds more than the injectable DNA (5 μg) vaccine, which makes it quite prohibitive to use it as a vaccine. Therefore, novel strategies for an oral vaccine against IHNV are needed, which are cost-effective for large-scale aquaculture populations. Although a licensed injectable DNA vaccine is available against IHNV [21], there are no oral vaccines for mass vaccination of fry.

The pro-inflammatory C5a, generated by the C5 component upon the complement system activation [22] was shown to induce chemotactic response in trout granulocytes [23] and carp neutrophils [24]. Research using recombinant C5a have also demonstrated its potential to chemoattract rainbow trout leucocytes *in vitro* [25], and *in vivo* by acting as a molecular adjuvant for soluble protein antigens [26]. In this research, a 77 amino acid-containing the rainbow trout C5a gene sequence was placed next to the sequence of IHNV G gene in the plasmid to subsequently produce a recombinant IHNV G-C5a protein in the host cells. We then tested its potential of eliciting a more efficient immune response in the vaccine-treated juvenile rainbow trout upon IHNV infection.

The baculovirus/insect cell expression system has been widely used for the expression of foreign genes, having a great potential for vaccine development [27–29]. The system is attractive because it is non-pathogenic towards vertebrates [30]. It provides for an abundant supply of the protein-of-interest due to the strong polyhedrin promoter [31]. In addition, the expressed proteins are post-translationally modified and correctly folded in most cases, as compared to *E. coli* expressed products. Recently, we demonstrated that oral vaccination of grass carp (*Ctenopharyngodon idella*) with baculovirus-expressed grass carp *Reovirus* (GCRV) proteins induces protective immunity against the GCRV infection [32].

Salmonids mount a complex immune response against pathogens, particularly when fighting early viral infections [33–35]. Type I interferons (IFN) orchestrate the transcription of the IFN antiviral cascade effectors to elicit the activation of IFN-stimulated genes (ISG), which strongly correlates with the viral burden during an infection [36–38]. In fish immunology, the sustained expression of IGSs, including of Mx proteins, is a common hallmark of the antiviral defense [39–42]. The differential expression of the T cell surface marker CD4, has been studied as an indicator of the stimulation of T helper lymphocyte (T_H) subsets differentiation to initiate an antiviral response [43,44]. T_H1-type cytokines are produced by $CD4^+$ T cells, following activation by APC, triggering the JAK/STAT cascade to directly potentiate the expression of IFN-γ and TNF-α family members, and promoting Treg development [45,46]. The differential gene expression of IgM H-chain was instead studied as a marker of an enhanced B cell adaptive immune response upon viral infection [47]. These immune response markers have been previously shown to be induced in the kidney and spleen of trout immunized with other experimental oral vaccines being tested against IHNV [20,48–50].

In this study, we produced recombinant IHNV G proteins in insect larvae and delivered these proteins orally to immunize juvenile rainbow trout. Thereafter we challenged immunized fish with IHNV to evaluate any protection elicited. Comparing data from experimental groups, we analyzed the differential survival rate and modulation in the transcription of marker genes for the activation of cellular and humoral responses.

2. Materials and methods

2.1. Generation of recombinant baculoviruses harboring IHNV G and IHNV G-C5a genes

The virulent IHNV 220–90 strain (M genogroup type) was cloned, and the complete nucleotide sequence was determined (GenBank accession no. GQ413939) [51]. Synthetic genes for IHNV G (1418 bp) and IHNV G-C5a (1649 bp) proteins were codon-optimized for expression in cabbage looper (*Trichoplusia ni*) insect larvae, and chemically synthesized with flanking restriction enzyme sites *Bam*HI and *Not*I at 5′ and 3′ ends, respectively (Biomatik). The IHNV G cassette was synthesized as a single gene construct comprising the ORF of IHNV G ectodomain (21aa - 452aa), followed by foldon sequence (29aa; for trimer formation), 6xHis residues to aid in purification, and a stop codon. Whereas the IHNV G-C5a cassette was also synthesized as a single gene construct, similar to the IHNV G cassette, except that it had a C5a gene sequence (77aa; used as an adjuvant) placed in frame between the G-ectodomain and foldon sequence. The plasmids containing these gene cassettes were used to prepare recombinant baculoviruses.

Procedures used to generate and characterize recombinant baculoviruses expressing the foreign proteins were essentially the same as described earlier [52], except that we utilized plasmid pAcGP67B as a transfer vector to clone IHNV G genes between unique *Bam*HI and *Not*I restriction sites and ProFold-ER1 baculovirus DNA for transfection (AB Vector) to improve the expression of IHNV glycosylated protein. Representative baculoviruses harboring the IHNV G and IHNV G-C5a genes and expressing respective proteins were selected and stored at −80 °C.

2.2. Production of IHNV-specific recombinant proteins in insect larvae

We propagated recombinant baculoviruses in Sf-9 cell cultures to prepare the seed virus, and then submitted to a company (Allotropic Tech) that produces recombinant proteins in *T. ni* insect larvae on a contract basis. This company uses proprietary techniques and delivers freeze-dried larvae (FDL) powder of uniform sized particles that contains the recombinant target antigens. Insect larvae were infected with IHNV G and IHNV G-C5a recombinant baculoviruses and harvested 72 h post-infection (hpi). We acquired over 200 g of FDL powder for IHNV G and IHNV G-C5a samples, which were used to characterize and quantify IHNV-specific proteins, before formulating oral vaccine feeds. These FDL samples containing recombinant IHNV antigens were irradiated to inactivate the baculovirus and stored at −80 °C until use.

For western blotting analysis, larval homogenates were prepared in PBS buffer with protease inhibitors. The crude homogenate and the supernatant obtained after centrifugation were separated by SDS-PAGE on a 4–15% gradient gel, blotted onto nitrocellulose membrane, reacted with anti-histidine (6xHis) antibody, and detected with alkaline phosphatase conjugate and BCIP/NBT color development reagents (BioRad).

2.3. Determination of recombinant proteins concentration in larvae powder

Larvae powders containing IHNV G or IHNV G-C5a proteins were sonicated in lysis buffer containing 50 mM Tris–HCl, pH 8.0/0.5 M NaCl/protease inhibitors (cOmplete EDTA-free, Roche Diagnostics). Each lysate was centrifuged at 27,000 g for 30 min. The target proteins present in the pellets were used for further purification to determine the protein concentration. Pellet was resuspended in 50 mM Tris–HCl, pH 8.0/6 M guanidinium chloride and subjected to sonication. After centrifugation, as above, the supernatant was passed through a HisPur Cobalt spin column (Thermo Scientific) that had been pre-equilibrated

with 50 mM Tris–HCl, pH 8.0/8 M urea/5 mM imidazole and eluted in 50 mM Tris–HCl, pH 8.0/8 M urea/100 mM imidazole. Aliquot samples of purified IHNV G and IHNV G-C5a were fractionated by 12% SDS-PAGE in parallel with a series of known amount of bovine serum albumin (Thermo Scientific) and stained with GelCode Blue Stain (Thermo Scientific). IHNV G and IHNV G-C5a protein concentrations were determined by comparing their band density with the albumin standards. To determine IHNV-specific protein in the larvae powder, a known amount (~20 mg) of powder was lysed in a certain volume of Laemmli Buffer (BioRad). Serially diluted amounts of the lysate were fractionated by 12% SDS-PAGE in parallel with a known amount of IHNV G standard, transferred onto a polyvinylidene fluoride membrane and probed with mouse anti-HisTag antibody, followed by horseradish peroxidase-conjugated goat-anti-mouse IgG. The membrane was treated with Clarity Western ECL substrate (BioRad), and an image was taken by ChemiDoc Touch Imaging System (BioRad). IHNV G and IHNV G-C5a contents were determined by comparing their band densities with IHNV G standards. The amount of IHNV G and IHNV G-C5a proteins were determined to constitute 3.2% of the larvae powder.

2.4. Preparation and evaluation of IHNV oral vaccine feeds

Formulation and evaluation of the IHNV oral vaccine feeds was carried out by the Riverence Provisions, Buhl, Idaho, USA on a contract basis. The immunization feeding trial was conducted comparing 3 test feedings; Control feed, IHNV G, and IHNV G-C5a test feeds, which were prepared by adding FDL protein powder to commercial feed at 4% inclusion, and top-coated with unflavored gelatin binder. Roughly 4 kg of each test diet was needed to feed fish twice for two weeks periods with recombinant proteins. At 10 week post-immunization, approximately 14.22 mg of IHNV-specific protein would be consumed by each fish, which should not pose a concern for immune tolerance [53].

2.5. Immunization and viral infection challenge of juvenile rainbow trout

Research-grade pathogen free rainbow trout were reared to appropriate size in a research aquatic biosafety level 2 wet-lab, using flow through, filtered, UV-irradiated water at 15 °C. Upon arrival to the lab, fish were allocated to three 100-gallon (379 L) tanks (Group A, Group B, and Group C). They were held off feed 1 day after stocking in the lab, and then fed with Control Feed of commercial feed for 2 weeks prior to the start of any treatment. Trout were primary immunized for 2 weeks using experimental feedings, followed by 4 weeks with regular feeding. A boosting immunization was given for 2 weeks using again experimental feedings, followed by 4 weeks with regular feeding (up to week 14). Respectively 10 and 4 weeks after each immunization feeding, fish were challenged with IHNV by immersion. Fig. 1 illustrates the experimental design.

On week 15, fish (average TBW 20 g) from each group were moved to triplicate challenge buckets (30 fish/bucket) containing a static bath with 1.3×10^5 PFU/mL of WT 331–16 IHNV (M genogroup) for 1 h, thereafter were allocated to their respective tanks. For the sham-exposed group the same procedure was used, but pouring into the tanks an equal amount of water and sterile transport medium used to suspend the virus (L15, Gibco). Fish were fed regular diet and monitored daily for morbidity and mortality for 15 days (till experiment termination). At 0 (before the infection challenge), 7, and 14 days post infection (dpi) or post sham-infection (dps), spleen from 6 fish from each treatment group (2 fish from each replicate tank) were collected and preserved individually in RNAlater (Invitrogen), kept at RT for 24 h and stored in −80 °C freezer until they were processed for RNA extraction.

The *in vivo* viral infection challenge experiment was conducted in compliance with guidelines provided by the Guide for the care and use of laboratory animals [54], and the U.S. Public health service's policy on the humane care and use of laboratory animals [55].

2.6. Total RNA extraction, quality check and cDNA synthesis

Total RNA was extracted from 100 mg of spleen tissue, homogenized with 1.4 mm ceramic beads (Fisherbrand), using Trizol-chloroform phase extraction , as described previously [56]. RNA pellets were dissolved in sterile 50 μL TE buffer (10 mM Tris and 1 mM EDTA; pH 8.0). RNA purity and concentration were determined using Epoch Microplate Spectrophotometer (BioTek). RNA samples were stored at −80 °C until use.

Total RNA specimens were reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega), following the manufacturer's instruction. Each reaction contained 1.5 μg of RNA, 1 μL of random hexamer primers, 1 μL of Oligo dT primers, 1 μL 10 mM dNTP, and PCR grade water to 10 μL final volume. Reverse transcription was carried out in a thermal cycler (Eppendorf), following the manufacturer's instruction. Each cDNA specimen was diluted with TE buffer (pH 8.0), and stored at −20 °C until use.

2.7. Real-time qPCR and gene expression screening

Reverse transcription quantitative PCR (RT-qPCR) was performed to quantify the individual viral burden, targeting the IHNV N gene, and to measure the transcription of a selected reference gene and of immune relevant genes using primers designed to target rainbow trout mRNA. All oligonucleotides and cycling conditions used in this study are listed in Table 1. A 10 fold dilution series of each purified amplicon was used to generate a standard curve for detection quantification.

IHNV N burden was measured using the TaqMan real-time PCR assay advised by WOAH [9], with the primer pair IHNV N 796F and 875R and the FAM-labelled probe IHNV N 818T [57], modified with Onyx Quencher A (MilliporeSigma) (Table 1). TaqMan™ Fast Advanced Master Mix (Applied Biosystems) was used following the thermal cycling conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

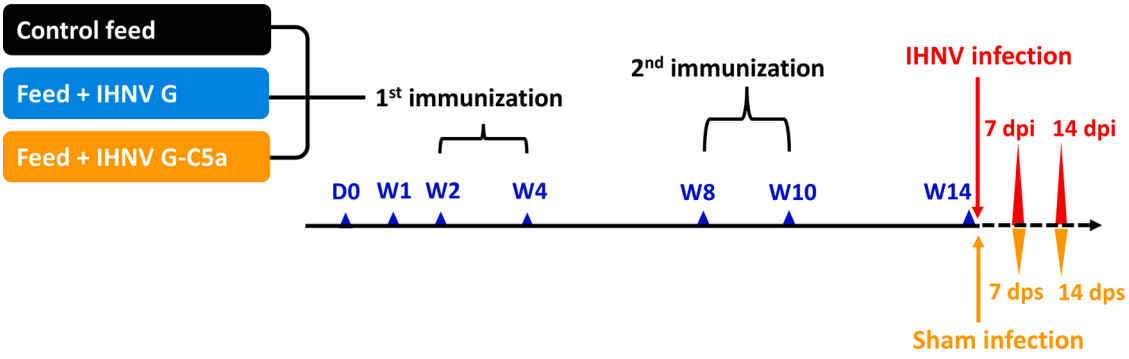

Fig. 1. - Experimental design of the immunization feeding trial followed by IHNV infection challenge in juvenile rainbow trout (*Oncorhynchus mykiss*).

Table 1
- Oligonucleotides and cycling conditions used in this study in juvenile rainbow trout (*Oncorhynchus mykiss*) spleens.

Target	Forward primer sequence: 5′–3′	Reverse primer sequence: 5′–3′	Amplicon size (bp)	*Ta* (°C)	Mt (°C)	Primer pair efficiency (%)	Source
IHNV Nucleocapsid	AGAGCCAAGGCACTGTGCG 6FAM-TGAGACTGAGCGGGACA-OQA	TTCTTTGCGGCTTGGTTGA	80	60	*n.a.*	97	[57]
EF-1α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	327	63	88	93	[58]
Type I IFN-a	CTGTTTGATGGGAATATGAAATCTGC	CCTGTGCACTGTAGTTCATTTTTCTCAG	193	64	83	91	[58]
Mx-1	CCTCCTGAAATCAGCGAAGAC	GAGTCTGAAGCATCTCCCTCTG	365	62	86	92	[58]
CD4	GTGTGGAGGTGCTACAGGTTTTTTC	ATCGTCACCCGCTGTCTGTG	396	64	87	99	[58]
IgM H membrane	CCTACAAGAGGGAGACCGATTGTC	GTCTTCATTTCACCTTGATGGCAGT	168	62	85	97	[58]

The transcription of each rainbow trout gene was measured using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), using the specific exon-skipping primers indicated in Table 1, and following the general thermal cycling conditions: 95°C for 10 min; 45 cycles of 95°C for 15 s, *Ta*°C (primer pair specific, see Table 1) for 60 s for fluorescence detection. The specific melting temperature (Mt) for each amplicon was measured with a melting curve, from 60 °C to 95 °C at every 0.5 °C per *sec*.

Each biological sample was tested by qPCR using 2 technical duplicates in a QuantStudio 3 Real-Time System (Applied Biosystems). The average target concentrations between technical duplicates from each specimen were extrapolated, calculating the average Quantification Cycle (Cq value), using Gene Expression Analysis software (Applied Biosystems). The specific gene transcription levels were normalized to the deterction of a rainbow trout reference gene, Elongation Factor 1α (EF-1α), quantified from the same cDNA specimen [20,50,60,61]. Data are presented as fold change, relatively to the expression levels in each control group, the uninfected fish sampled at day 0, which was considered as the basal expression level. The transcription levels retrieved from each fish were anchored to the lowest value in each data set followed by log2 transformation, as described previously [56], before statistical analysis.

2.8. Statistical analysis

The comparison of the survival rate between experimental groups over time, was calculated using Kaplan-Meier survival analysis (Log-Rank test), followed by a pairwise multiple comparison. The individual viral burden, assessed targeting the IHNV N gene, was compared across experimental groups using one-way ANOVA. The average expression levels of immune genes across groups were analyzed using two-way ANOVA, followed by Tukey post hoc test. Differences were considered as statistically significant where $p<0.05$. The degree of correlation between individual pathogen burden and specific gene expression was calculated by parametric correlation analysis. Pearson product-moment correlation coefficient r was considered significant with $p<0.05$ (2-tailed). Statistical analyses were performed using GraphPad Prism 9 (Dotmatics).

3. Results

3.1. Expression of recombinant IHNV G proteins in insect larvae

The expression of IHNV glycosylated proteins produced in *T. ni* insect larvae, upon infection with recombinant IHNV G and IHNV G-C5a baculovirus, was assessed by western blot analysis. Cellular proteins were separated by denaturing polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with anti-histidine (6xHis) antibody. Fig. 2 shows the bands produced by reactivity of His-tagged IHNV G proteins with anti-histidine monoclonal antibody after immunostaining. Two top bands (marked with arrow) in lanes 1–2 of about 61.3 kDa represent IHNV G-C5a protein in the supernatant and pellet fraction of insect lysate, respectively, whereas the bands below could be of non-glycosylated proteins. Similarly, two top bands (marked with arrow) in lanes 3–4 of about 56.5 kDa represent IHNV G glycosylated protein in the supernatant and pellet fraction, respectively, whereas the bands below of about 52.3 kDa could be of non-glycosylated protein. These results indicate that IHNV G proteins were successfully expressed in *T. ni* larvae.

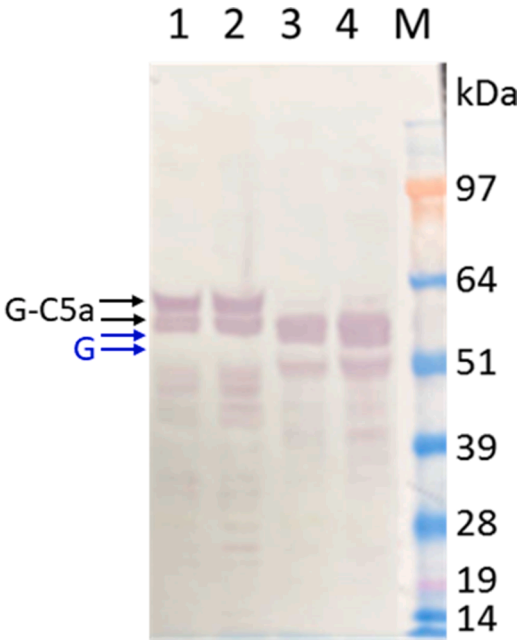

Fig. 2. - Western blotting assay of IHNV proteins produced by recombinant baculovirus-infected insect larvae. Lanes 1–2: respectively supernatant and cell pellet of IHNV G-C5a baculovirus-infected insect larvae lysate; lanes 3–4: respectively supernatant and cell pellet of IHNV G baculovirus-infected insect larvae lysate, lane M: SeeBlue Plus2 Pre-Stained Standard (Thermo Fisher).

3.2. Fish survival after experimental infection

The trout mortality upon IHNV infection started at 3 dpi in the control feeding group, followed at 4 dpi in the IHNV G immunized and the IHNV G-C5a immunized group (Fig. 3.A). After one week post viral infection, the cumulative mortality was lower in the immunized groups, when compared to the control feeding group, respectively 31% lower in the IHNV G immunized group and 49% lower in the IHNV G-C5a group. The final cumulative mortality reached at 15 dpi was 97.6% in the control feeding group, but 9.2% lower in the IHNV G immunized group (88.4%) and 15.9% lower in the IHNV G-C5a immunized group (81.7%). No mortality was recorded in the sham-infection group (not shown in the graph). Significant differences were retrieved between the survival probability in the groups immunized with recombinant IHNV G and IHNV G-C5a proteins, when compared to the control feeding group (Fig. 3.B). A significant difference of the survival probability was also seen between the IHNV G and IHNV G-C5a immunized groups ($p = 0.04$) upon Kaplan-Meier survival analysis.

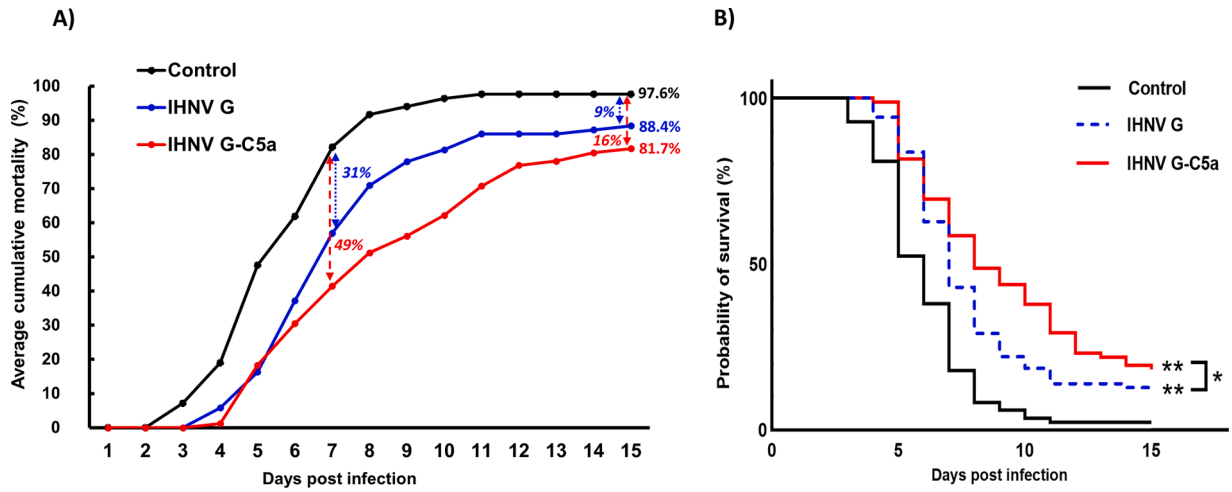

Fig. 3. - Mortality after feeding trial and infection challenge across experimental immunization groups. (A) Average cumulative mortality; (B) Kaplan-Meier probability of survival analysis. Statistical difference between treatments is shown: * $p<0.05$; ** $p<0.01$.

3.3. Viral burden analysis

The individual IHNV burden was measured in spleens from immunized fish, sampled at 7 and 14 dpi following the viral infection challenge. No significant differences were found across treatment groups from each sampling day (Fig. 4). At 14 dpi the viral burden decreased in the spleen of trout from all treatment groups. IHNV was not detected in any of the sham infection groups.

3.4. Constitutive immune gene transcription in immunized trout

To assess any immunomodulatory effect elicited by the feeding immunization treatments, we studied the baseline transcription of selected immune relevant genes in spleens sampled from fish in the sham infection group at 0, 7 and 14 dps. No significant difference was found across groups sampled on the same day for the antiviral response markers, Type I IFN-a, and Mx-1 (Fig.5A and B). The constitutive expression level of CD4 decreased during the experiment, becoming significantly lower at 7 dps in the control feed group and in all groups at 14 dps, when compared to day 0 (Fig. 5.C). The constitutive expression of IgM H was significantly higher in the IHNV G-C5a immunized group at 0 dps in comparison to the other groups, although not statistically significant due a high individual variance, and was instead significantly downregulated at 14 dps in the same treatment group (Fig. 5.D).

3.5. Comparative modulation of antiviral markers

To assess the antiviral protection conferred by each feeding immunization treatment, we studied the transcription modulation of selected markers involved in the host antiviral response against IHNV infection. A strong induction of Type I IFN-a was detected across the groups at 7 dpi, when compared to their sham-infection counterparts, although the IFN induction was much more contained at 14 dpi (Fig. 6.A). Type I IFN-a induction was more strongly correlated to the viral burden in the groups immunized with both IHNV G and IHNV G-C5a proteins, rather than in the control group (Fig. 6.C). A strong induction of Mx-1 was detected across the groups at 7 dpi, when compared to their sham-infection counterparts (Fig. 6.B). At 14 dpi Mx-1 was still induced but showing a stronger upregulation in the control group. Mx-1 transcription positively correlated to the viral burden, although the stronger correlation was found in the IHNV G treated group ($r = 0.87$) (Fig. 6.D).

3.6. Comparative modulation of cellular response markers

To assess the cellular response modulation upon each feeding immunization treatment, we studied the transcription of selected markers

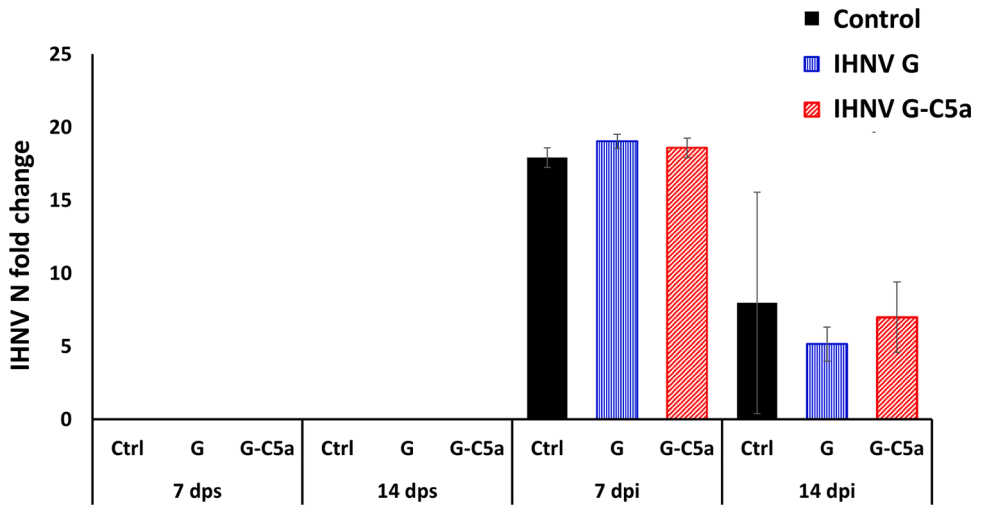

Fig. 4. - IHNV burden assessment of immunized rainbow trout exposed to a viral infection challenge. Data from individual spleen cDNA are normalized to the expression of a reference gene, EF-1α, and presented as group means ±SE ($n = 6$, except in the control group at 14 dpi $n = 2$).

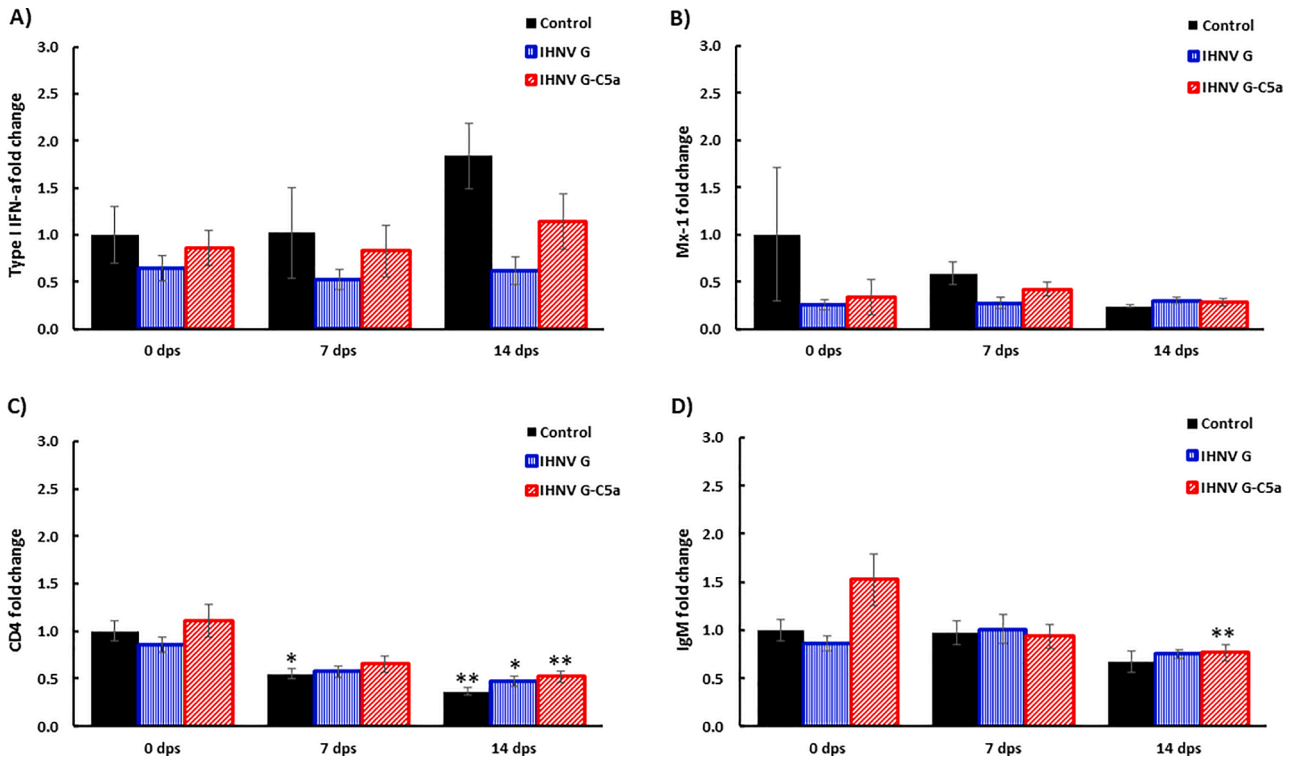

Fig. 5. - Constitutive expression profiles of selected rainbow trout immune related genes upon sham infection challenge. (A) Type I IFN-a; (B) Mx-1, (C) CD4; (D) IgM membrane H chain. Data are normalized to the expression of a reference gene, EF-1α, and presented as group means ±SE ($n = 6$). Significant differences across groups are indicated relatively to 0 days post sham-infection (dps): *$p<0.05$; **$p<0.01$.

involved in the protection against IHNV infection. An initial strong downregulation of CD4 was detected across the groups at 7 dpi, when compared to their sham-infection counterparts (Fig. 7.A). Although at 14 dpi CD4 was instead upregulated in all treatment groups, but significantly only in the IHNV G treated group. CD4 modulation was strongly negatively correlated to the viral burden in all experimental groups (Fig. 7.C). The transcription of IgM membrane H chain was significantly downregulated across the groups either at 7 or 14 dpi (Fig. 7.B). No significant correlation was found between the transcription of IgM membrane H chain and the individual viral burden (Fig. 7. D).

4. Discussion

To avoid the stress-causing, laborious and time-consuming issues caused by injection, a vaccine delivered by oral route is still a good option to explore in aquaculture [62,63]. This becomes poignant when juvenile fish are highly vulnerable to a viral infection, as to IHNV during the first two months of their life, but still too small to receive any injection [1,15,64,65]. In this study, oral delivery of new recombinant IHNV G vaccines produced in cabbage looper insect larvae conferred a moderate survival improvement to juvenile rainbow trout upon IHNV immersion infection challenge (Fig. 3.B). Despite fish remained vulnerable to the IHNV infection (Fig. 4) the onset of the bulk mortality was delayed, and the final cumulative mortality reached lower levels in the immunized groups, when compared to the control group (Fig. 3.A). By the experiment termination (at 15 dpi) the mortality was 15.9% lower in the IHNV G-C5a immunized group, and 9.2% lower in the IHNV G immunized group, when compared to the 97.6% mortality caused by the IHNV infection to the control feeding group. These data indicate an improved survival rate in juvenile rainbow trout conferred by the oral immunization that could be beneficial in aquaculture to reduce mass mortality events happening upon an accidental introduction of IHNV in fish hatcheries. Although, the evidence we retrieved is not strong enough to provide an adequate protection level that could justify the commercialization of these recombinant compounds for cost-efficient vaccination strategies. Several reasons could have caused this result, including the degradation of antigen protein in the trout digestive tract, an insufficient amount of the antigen delivered, or a high IHNV challenge dose. The IHNV G antigens may have been degraded before their intake in the hind gut. An anti-IHNV DNA vaccine, encapsulated into alginate microspheres and orally fed to rainbow trout, showed evidence of inducing immune response in kidney and spleen, however conferring only partial protection against IHNV infection [20]. It is not surprising that the unencapsulated oral fed vaccine failed in generating a much stronger or complete protection against IHNV infection in juvenile rainbow trout, which are highly vulnerable to the infection and of virulent M genogroup IHNV [1,64,66]. To further investigate the efficiency of vaccines that could be delivered orally, a better encapsulation of the antigens or antigen-generating vectors should be considered.

The analysis of the constitutive transcription of selected immune gene markers, in spleens of sham-infected fish, showed mild beneficial modulation happened in the immunized group. This might have been caused by a delayed sampling; indeed, the constitutive transcription was assessed from sham-infected fish sampled 4 (0 dps), 5 (7 dps), and 6 (14 dps) weeks after the completion of the boost immunization. In other IHNV vaccine development studies, these sampling points were mostly located 3 to 18 days after the boost immunization, thus showing an increase in the immune gene expression [67,68]. However, in an experimental vaccination study carried out in grass carp against GCRV infection, the up regulation of immune related genes was measured from 21 to 42 days post immunization [32]. In our study, the expression of the IgM antibody response marker, showed a higher constitutive transcription in fish immunized with IHNV G-C5a protein at 0 dps, when compared to the same group at 14 dps. It remains questionable if our oral vaccine treatments may have elicited any appreciable induction of immune related genes shortly after the end of each immunization cycle. Although the main goal of this study was to assess any protection

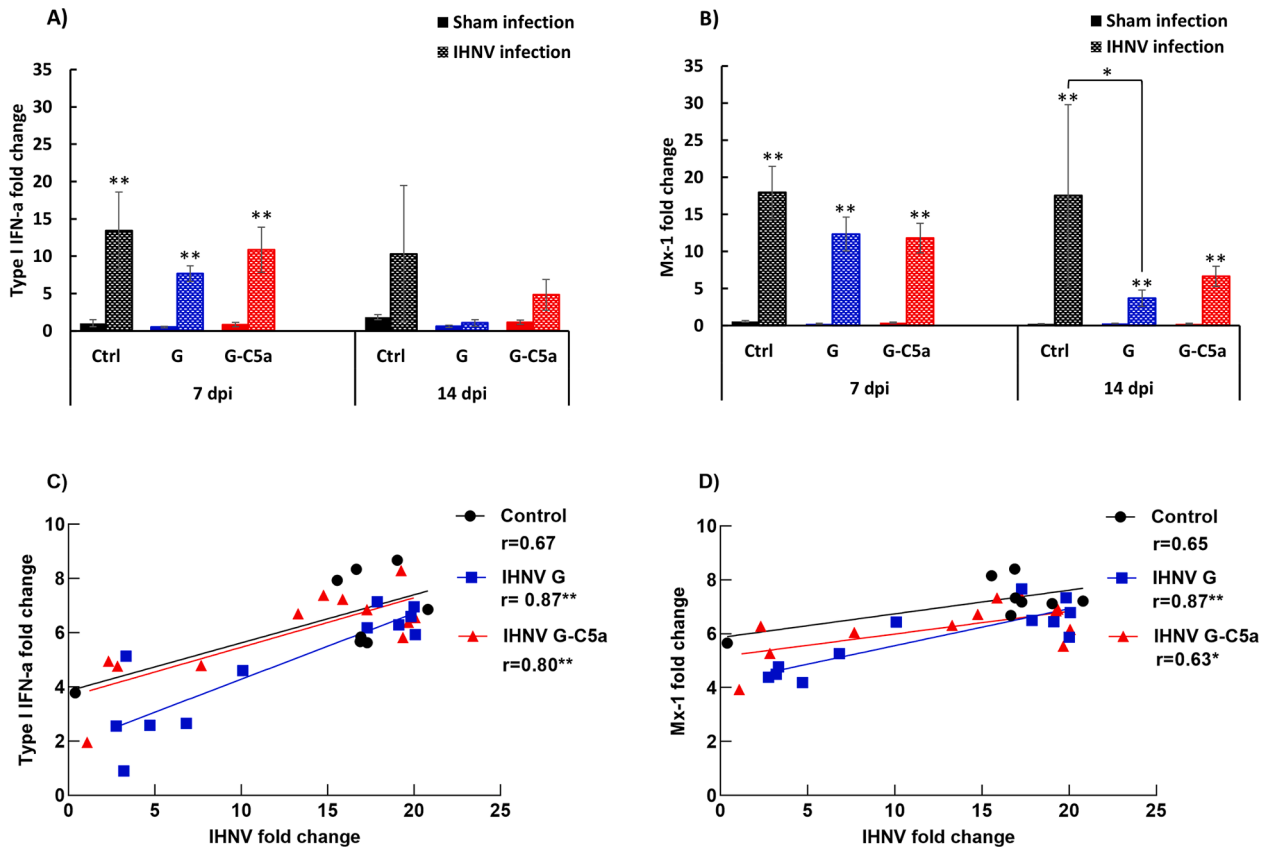

Fig. 6. - Gene transcription modulation of selected rainbow trout immune related genes upon IHNV infection. Fold change across experimental treatment groups for Type I IFN-a (A), and Mx-1 (B). Data are normalized to the expression of a reference gene, EF-1α, and presented as group means ±SE ($n = 6$). Significant differences across groups are indicated relatively to their respective sham-infection groups. Correlation analysis between the individual gene transcription of Type I IFN-a (C), or Mx-1 (D), and IHNV N are used as a proxy of to measure the individual viral burden. Pearson correlation r coefficients are indicated. Statistical relevance is indicated: *$p<0.05$; **$p<0.01$.

conferred by the immunization strategies upon viral infection, thus simulating the recombinant vaccine administration to juvenile trout in a fish hatchery.

Type I IFN and Mx-1 are frequently used as markers to assess the modulation of the innate immune response upon viral infection or vaccine administration [15,32,67]. In our study, their expression trends were comparable across treatments groups and sampling points. Mx-1 was significantly induced at 7 dpi in all three different feed treated groups, but at 14 dpi decreased in both the IHNV G and IHNV G-C5a treated groups in comparison to the control fed group (Fig. 6.B). This result may provide an indication that both experimental vaccines might have played a modulatory role in shortening the IHN pathogenesis, in line with the delayed and lower mortality retrieved upon these treatments (Fig. 3.A). Furthermore, also the IHNV burden showed decreasing trends in spleens of immunized fish at 14 dpi, although with a high individual variance (Fig. 3.B). Further evidence supporting the occurrence of stronger immune response in immunized fish can be retrieved from the transcriptional analysis of the T cell co-receptor CD4 (Fig. 7). Immunized trout showed higher CD4 expression levels at 14 dpi, although a statistical relevance (when considering $p<0.05$) was only achieved in the IHNV G protein treated fish. The correlation analysis showed that immunized fish with both compounds tend to have higher CD4 gene transcription levels. CD4 expression modulation patterns may indicate that our immunization treatments improved the differentiation of the $CD4^+$ T helper subtypes to better coordinate the antiviral response. The higher expression of CD4 gene in the control fish group at 0 dps, when compared to 7 and 14 dps, and the similar trend seen in the vaccinated fish groups, might indicate that some experimental factors may have been influencing all the groups before the infection challenge. Although, the lack of a beneficial IgM modulation across treatments both 7 and 14 dpi, might suggest that the antibody response should have been measured at a later sampling point. Strong antibody responses against either viral haemorrhagic septicaemia virus (VHSV) and IHNV are generally seen from 4 wpi [69,70].

The C5a protein was shown to induce chemotactic response in rainbow trout granulocytes [23], and in carp neutrophils [24]. This C5a protein has chemoattractant properties for leucocytes from peripheral blood and head kidney, and can induce respiratory burst in the same cell type *in vitro* [25]. In another study, it was shown that when purified recombinant C5a-IHNV G protein was injected intraperitoneally into rainbow trout, it induced a stronger adjuvant effect and stronger IgM antibody level than the IHNV G protein alone [26]. In our study the IHNV G-C5a immunized group had a considerably delayed mortality and had a better survival rate at 15 dpi. Although, we did not see a strong adjuvant effect played by C5a added to IHNV G on the modulation of the immune response markers, when compared to the IHNV G protein immunized fish.

Vaccine dosage is an important concern in the attempt to stimulate an efficient immune response against the target pathogen [71,72]. Dosage may have been another reason for our experimental immunization strategy failure. We provided two rounds of two-week immunization with feedings containing 4% of lyophilized cabbage looper larvaal powder. In a similar research approach, lyophilized silkworm larval powder was delivered orally to protect grass carp against GCRV infection. A protection with 56% relative percent survival was achieved feeding the fish with 5% integration for 6 weeks without boosting [32]. Notably, similar research in grass carp showed that 1% feeding integration with lyophilized silkworm powder could generate even higher

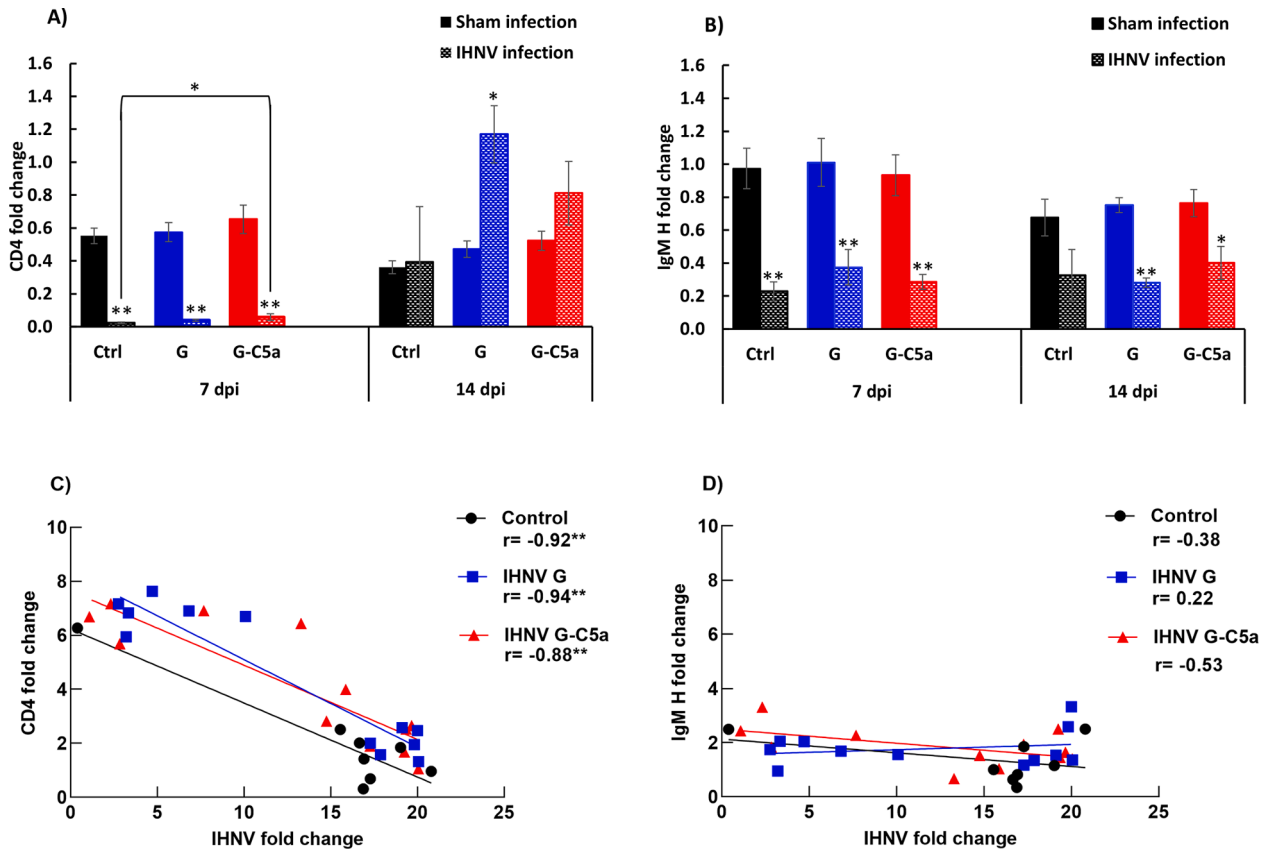

Fig. 7. - Gene transcription modulation of selected rainbow trout immune related genes upon IHNV infection. Fold change across experimental treatment groups for CD4 (A), and IgM membrane H chain (B). Data are normalized to the expression of a reference gene, EF-1α, and presented as group means ±SE ($n = 6$). Significant differences across groups are indicated relatively to their respective sham-infection groups. Correlation analysis between the individual gene transcription of CD4 (C), or IgM membrane H chain (D), and IHNV N are used as a proxy of to measure the individual viral burden. Pearson correlation r coefficients are indicated. Statistical relevance is indicated: *$p<0.05$; **$p<0.01$.

antibody titer against GCRV than 5 or 10% silkworm lyophilized powder did at different time points after oral immunization [73]. A suitable proportion of the target antigen from the selected antigen-producing host might need to be further elucidated in the similar research models in the future to test the efficiency of the vaccines in question. On the other hand, most IHNV vaccine research that applied immersion as the viral infection route used 1×10^3 to 1×10^4 PFU/mL in their research, whereas it was 1.3×10^5 PFU/mL in our study which caused 97.6% mortality in the unvaccinated group. Finally, the relatively higher viral dose might be also a reason that caused high mortality in fish, which might have overwhelmed the vaccine generated immunity [74].

Concluding remarks

The lyophilized cabbage looper insect larvae powder used to deliver IHN vaccines in feed resulted inadequate to immunize rainbow trout against the IHNV infection. Nevertheless, this new oral vaccine delayed the mortality and moderately influenced the transcription of selected immune related genes upon IHNV infection. Experimental design avoiding the experimental issues discussed would help to better assess the efficiency of a new vaccine in future trials. To assess how new oral vaccines may be effective to mitigate IHNV pathogenesis by modulating the host immune response towards IHNV exposure, further studies are needed.

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.

Data Availability

Data will be made available on request.

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