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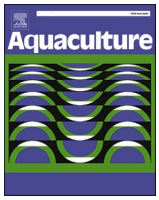

An encapsulation-free oral subunit vaccine for VHSV: Inducing robust antiviral and antibody responses

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ABSTRACT

Viral haemorrhagic septicaemia (VHS) is a viral disease that causes high mortality in numerous teleost species, both farmed and wild. A commercial vaccine against the VHS virus is not yet available. Due to the difficulty and stress of early-stage injection, the oral route would undoubtedly be the optimal vaccination route in terms of animal welfare and handling costs. However, oral administration has drawbacks, primarily weak immune protection and vaccine degradation, which in most cases requires additional encapsulation. Here we present a recombinant protein subunit vaccine against VHSV that is highly stable, does not require encapsulation, and stimulates both local and systemic antiviral responses after oral intubation. Additionally, it activates the adaptive immune response, inducing the production of specific and neutralizing antibodies when administered either by intubation or incorporated into fish feed. The vaccine consists of VHSV glycoprotein G fused with an interferon-gamma (IFNγ) domain from *Oncorhynchus mykiss*, overexpressed in *Escherichia coli* and purified as a highly structured nanostructured biomaterial (nanopellets, NPs). These NPs were internalized by the rainbow trout intestinal epithelial cell line (RTgutGC) and rainbow trout head kidney macrophage (RT-HKM) cells and were fully functional triggering antiviral and inflammatory gene expression and activating the interferon signaling pathway. To evaluate whether VHSV-IFN^{NP} could be absorbed in the gut and induce a local and systemic immune response, trout fry were intubated at two doses, 30 mg/kg fish and 125 mg/kg fish. Analysis of hindgut-sorted leukocytes revealed two populations—myeloid and lymphoid—the latter showing a strong antiviral response at the higher dose. Consistent with this, we observed in the head kidney (HK) of fish intubated with VHSV-IFN^{NP}, a clear antiviral gene response. Interestingly, fish treated with VHSV-IFN^{NP} at 125 mg/kg fish and 500 mg/kg fish administered by intubation and orally in the diet, respectively, showed an increase in specific and neutralizing antibody titres 30 days after vaccination, demonstrating its ability to induce a robust adaptive response.

1. Introduction

Viral haemorrhagic septicaemia (VHS) is a viral disease that has been detected in about 80 different fish species in both marine and freshwater environments and known to cause high mortality in different species, both farmed and wild. The virus is widespread throughout the world, with a higher incidence in northern hemisphere aquaculture (Norwegian Fish Health Report, 2022 World Organization for Animal Health [OIE], 2009). VHS symptoms include protruding eyes, distended abdomen, bleeding, and anemia. High mortality outbreaks are a

Abbreviations: NPs, Nanopellets; VHSV, viral haemorrhagic septicaemia virus; IFNγ, interferon gamma; iRFP, near-infrared fluorescent protein; ip, Intraperitoneal; NAb, Neutralizing antibody; dd, degree-days.

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problem for rainbow trout farms that control them by destruction of all fish in the infected location. VHS is a notifiable disease in the EU (category C + D + E). Therefore, EU countries are obliged to conduct routine clinical inspections as part of a surveillance programme in Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss*) farms (Norwegian Fish Health Report, 2022). Although research on vaccine development for VHSV has been ongoing for more than four decades, there is still no vaccine in the market. Candidate vaccines include inactivated vaccines (Tamer et al., 2021; Kim et al., 2016), attenuated live vaccines (Adelmann et al., 2008; Kim and Kim, 2011; Kim et al., 2011; Kim et al., 2015), DNA-based vaccines (Lorenzen et al., 1998; Fernandez-Alonso et al., 2001; Byon et al., 2005; Lorenzen et al., 2009; Einer-Jensen et al., 2009; Cuesta and Tafalla, 2009; Bela-ong et al., 2015; Standish et al., 2016; Sepúlveda et al., 2019), and recombinant vaccines both using prokaryotic and eukaryotic expression systems (Lorenzen et al., 1993; Estepa et al., 1994; Lecocq-Xhonneux et al., 1994; Thwaite et al., 2018; Naderi-Samani et al., 2020).

Among the new generation of vaccines, DNA vaccines have often shown good levels of protection against viral infections due to their ability to induce strong and long-lasting cellular and humoral immune responses. However, there are currently only two DNA vaccines available in the market: Apex-IHN against IHNV marketed in Canada, and Clynav against alpha virus type 3 (SAV) which is licensed to be administered to Atlantic salmon in Europe since 2017. The procedure for marketing a veterinary DNA vaccine in Europe requires the resolution of several issues, primarily those concerning the safety of the vaccinated fish, the consumer concerns, and the environmental impact. The question of whether a DNA-vaccinated fish is considered as a genetically modified organism (GMO) is crucial for consumer acceptance and environmental safety (Ma et al., 2019; Collins et al., 2019). In this context, subunit vaccines offer a significant advantage as they do not contain live or complete pathogens that may replicate within the host. This is particularly true for recombinant proteins, where specific immunogenic proteins are produced using different expression systems such as yeast, transgenic plants, insects, cell cultures or bacteria. The subunit vaccines offer a number of advantages: they have immunological properties that are chemically well-defined and stable; they can be designed to stimulate specific immune responses; they are free of infectious components and have no residual toxicity or risk of toxicity reversion; they are easier to manufacture and allow the development of multivalent vaccines; and they can be freeze-dried, allowing non-refrigerated transport and storage, and they can be extruded, thus allowing an oral delivery strategy (Hansson et al., 2000; Du et al., 2022).

Currently, most of the commercial fish vaccines (mostly inactivated vaccines) are administered by injection. While injectable vaccines are known to provide the best protection, they are also associated with laborious handling and stress for the fish. Furthermore, water-in-oil (w/o) injectable vaccines are associated with local side effects such as inflammation, adhesion, pigmentation and necrosis, and the appearance of granulomas in essential organs. Other relevant adverse effects include stunted fish growth, reduced carcass quality and spinal deformities (Midtlyng, 1997; Embregts and Forlenza, 2016; Tripathi and Dhamotharan, 2022). In this context, oral administration would undoubtedly be the optimal mode of vaccine delivery in terms of animal welfare and handling costs. To date, two oral subunit vaccines have been commercialized: one for IPNV using the *E. coli* expression system marketed in Norway (Aquavac® IPNV Oral), and the second, an oral vaccine against ISA marketed in Chile, which is currently included in a polyvalent format and administered *via* ip (BLUEGUARD® IPN + SRS + As+Vo + ISA).

One of the drawbacks of subunit vaccines is that they induce a weaker immune response because they are usually made with a single antigen and do not replicate in the host like live/attenuated vaccines, so they may need multiple or booster doses and the addition of adjuvants to improve and prolong the immunization (Hansson et al., 2000; Du et al., 2022). IFNs are highly expressed cytokines with potent antiviral properties and strong adjuvant characteristics able to induce cell-mediated immunity during adaptive immune responses. Two types of teleost IFNs have been identified: type I and type II (γ). Innate immunological defences against viral infection are activated by type I interferon (IFN), whereas type II (IFN) is more closely associated with enhancing cell-mediated immunity. It is known that all IFNs (IFNα, β, γ and λ) induce an increase in surface expression of class I MHC antigens, whereas class II antigens are predominantly stimulated by IFNγ, with little or no effect of IFNα and IFNβ (Secombes et al., 2009; Toporovski et al., 2010; Zou and Secombes, 2011). In addition to its ability to enhance antigen presentation, IFNγ is involved in macrophage and T lymphocyte activation, cell proliferation, apoptosis, leukocyte trafficking and inflammation. More specifically, IFNγ plays a role in the differentiation of Th1 lymphocytes and the activation and differentiation of macrophages into M1-type macrophages, which are characterized by the production of pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO) (Pereiro et al., 2019). With all these attributes, IFNγ has been considered a major effector of immunity and has been used in the treatment of several human diseases (Castro et al., 2021). In fish, recombinant IFNs have been shown to have adjuvant properties based on *in vitro* models and their antiviral activity has been tested in different fish cell lines (Zou et al., 2005; Sun et al., 2011; Jung et al., 2012; Kuo et al., 2016; Bedekar et al., 2018; Morales-Lange et al., 2021). Furthermore, a combination of a nanostructured recombinant SVCV antigen and a recombinant zebrafish IFNγ has been shown to induce a strong immune response in ZFL cells and protected adult zebrafish against SVC virus when the vaccine is administered by ip injection (Rojas-Peña et al., 2022). In this context, the present study aims to evaluate a modular subunit vaccine containing the VHSV viral antigen fused to trout IFNγ, for oral administration to fish.

For oral vaccination, the vaccine is either pre-mixed with the feed during feed preparation, usually by extrusion, or mixed with the vaccine prior to feeding by homogenization with oil or vacuum coating (Makesh et al., 2022). Although the simplest method is to mix the vaccine with an oil matrix, both vacuum coating and, above all, extrusion allow the compound to remain homogeneous in the feed and available to be released in the fish's digestive tract with the same dynamics as the normal nutrients. However, both processes involve thermal and mechanical treatments at different pressures and temperatures (recommended above 70 °C) (Chaabani et al., 2020), which may affect the integrity of the proteins.

In this study, we evaluated the structural stability of the VHSV-IFN^{NP} subunit vaccine under extreme conditions of pH and temperature, and we also assessed its functionality in trout macrophages in *in vitro* assays and *in vivo* using both intubation and oral administration in trout fingerlings. To this end, we measured the local immune response in the gut and the systemic adaptive immune response in immune-relevant organs. Additionally, we evaluated the antibody titer 30 days post-administration *via* intubation and in-feed methods.

2. Materials and methods

2.1. Design, production and quantification of VHSV-IFN^{NP}

2.1.1. Viral strains and plasmids

Modular VHSV vaccines (VHSV^{NP} and VHSV-IFN^{NP}) were designed using the glycoprotein G sequence of viral haemorrhagic septicaemia virus (AA 252–449, Uniprot KB acc. no: P27662), developed previously by Thwaite et al. (Thwaite et al., 2018), plus an extra functional domain coding for recombinant interferon-gamma (IFNγ) of *O. mykiss* (AA 25–180, Uniprot KB acc. no: Q5QSL2_ONCMY). Clones were codon optimized for expression in *E. coli*, synthesized by GeneArt and subcloned into pET22b. Plasmid pET22b included a C terminal polyHis-tag. Plasmids were transformed into *E. coli* BL21 (DE3) (Novagen) by heat shock for recombinant protein expression. The near infrared fluorescent protein (iRFP^{NP}) cloned into pET22b (GeneArt), was used as a non-

relevant control protein.

2.1.2. Production and purification

The nanostructured recombinant proteins VHSV^{NP}, VHSV-IFN^{NP} and iRFP^{NP} were produced and purified as described previously (Thwaite et al., 2018), with some modifications detailed in (Rojas-Peña et al., 2022). *E. coli* was grown overnight (O/N) in LB medium (Gibco) with ampicillin (100 μg/ml, Sigma-Aldrich) and protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Panreac) for 4 h at 37 °C and 250 rpm. For purification, the cultures were subjected to an enzymatic and mechanical disruption as described before (Torrealba et al., 2016). Briefly, cell cultures were treated with lysozyme (1,5 μg/ml), protease inhibitor cOmplete EDTA-free Tablets, and phenyl-methanesulfonyl fluoride (0.4 mM PMSF), for 2 h at 37 °C and 250 rpm. Then suspensions were frozen (O/N) at −80 °C. After thawed, Triton X-100 was added (0.2 % *v*/v), and the solution stirred for 1 h at room temperature (RT). DNase I (1 μg/ml) and $MgSO_4$ (1 mM) were then added, and the cultures were shaken while incubating for 1 h at 37 °C. NPs were harvested by centrifugation at 15000 x*g*, 15 min at 4 °C and resuspended in buffer (50 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA) at 1/10 the original culture volume. Finally, additional freeze-thaw cycles were carried out to eliminate any remaining viable bacteria.

2.1.3. Quantification, and fluorescent labelling

VHSV^{NP}, VHSV-IFN^{NP}, and iRFP^{NP} were semi-quantified by Western Blot using an anti-His-tag antibody (Genscript A00186–100) and the final concentration calculated using Image Lab 6.0.1 (Bio-Rad). VHSV^{NP} and VHSV-IFN^{NP} were conjugated with Atto-488 NHS ester (Sigma-Aldrich) according to the manufacturer's instructions. The fluorescent VHSV^{NP} and VHSV-IFN^{NP} were used in flow cytometry and confocal microscopy. Labeling efficiency was determined with the Nanodrop ND-1000 (Thermo Scientific).

2.2. Morphometric characterization of VHSV-IFN^{NP}

Field Emission Scanning Electron Microscopy (FESEM) was used to determine the morphology and size of the nanopellets (NPs). Samples were prepared by resuspending VHSV-IFN^{NP} at 100 μg/ml in sterile mQ water and 20 μl were deposited onto silicon chips and air-dried (O/N). Images were taken with a FESEM (Zeiss Merlin) and analyzed with Fiji package (Schindelin et al., 2012). At least 60 nanoparticles were measured, and size distribution histograms were plotted using Past 4.04 software (Hammer et al., 2001).

2.3. Functional characterization of VHSV-IFN^{NP} subunit vaccine in vitro

2.3.1. Cell culture

The rainbow trout intestinal cell line RTgutGC (Rainbow Trout gut Guelph Canada) (Kawano, 2009) were cultured according to Brandts et al. (Brandts et al., 2020) at 20 °C under normal atmosphere, in medium L-15 + GlutaMAX (Gibco), 10 % heat-inactivated fetal bovine serum (FBS) (Gibco) and 1 % (*v*/v) antibiotic/antimycotic (Gibco). Rainbow trout head kidney macrophages (-HKM) were isolated from *O. mykiss* (~100 g body weight) following previously described procedures (Roher et al., 2011). The primary adherent culture was maintained in DMEM + GlutaMAX, 10 % heat-inactivated FBS and 100 μg/ml Primocin (Invitrogen) at 18 °C and 5 % CO_2. NPs uptake and gene expression assays were all performed at day 5 when monocytes were fully differentiated to macrophages.

2.3.2. Uptake of VHSV-IFN^{NP} by RTgutGC and HKM cells

To test the cellular internalization of the VHSV-IFN^{NP} subunit vaccine, fluorescent NPs (section 2.1) were added to RTgutGC and HKM cells at 70–80 % confluence after 2 h incubation in minimal medium (0 % FBS), at the doses and times listed below. For dose–response assays, the NPs was added at 2.5, 5, 10, 20 and 50 μg/ml and incubated for 12 h. For time-course assays, VHSV-IFN^{NP} were added at 10 μg/ml and incubated for 12 and 24 h. To compare the uptake between the modular vaccine that include the VHSV-G antigen and the modular vaccine that include the VHSV-G antigen combined with the rainbow trout IFNγ, VHSV^{NP} and VHSV-IFN^{NP} were added at 10 μg/ml and incubated for 16 h. After treatment, cells were trypsinized with 1 mg/ml Trypsin (Gibco), and sedimented by centrifugation at 300 ×*g* for 5 min at RT. Pellets were resuspended in PBS or minimal medium (Leibovitz's L-15) for flow cytometry (CytoFLEX LX), and at least 10,000 events were analyzed. Data was analyzed using FlowJo 10.4 (Leland Stanford University) and plotted with Prism 8.01 (GraphPad Prism). Confocal microscopy (Zeiss LSM 700) was used to further analyzed the interaction between NPs with living cells. Briefly, RTgutGC and HKM cells were seeded on Ibidi plates (μ-Dish 35 mm) and incubated at 20 °C under normal atmosphere and 18 °C, 5 % CO_2, respectively. The next day cells at 50–60 % confluence were placed in minimal media. After 2 h, VHSV-IFN^{NP} at 20 μg/ml was added and cells were incubated for 14 h at 18 or 20 °C. The cells were also stained with Hoechst (10 μg/ml) and CellMask Deep Red (5 μg/ml) (Life Technologies). Images were analyzed with Imaris v8.2.1 (Bitplane).

2.3.3. Gene expression analysis in HKM stimulated with VHSV-IFN^{NP}

Gene expression analysis was performed in HKM cells, due to its important role in the innate immune response and in the activation of the adaptive response. Cells were obtained as explained in section 2.3.1, cultured in minimal medium for 2 h and finally stimulated for 14 h with NPs and the corresponding controls. The following concentrations were used: 20 μg/ml of VHSV^{NP} and VHSV-IFN^{NP}, 20 μg/ml of iRFP^{NP} as immunogenically irrelevant protein, 10 μg/ml of LPS from *E. coli* O111: B4 strain (Sigma-Aldrich), 10 μg/ml of poly(I:C) (Sigma-Aldrich) as a viral dsRNA mimic, and control cells with no stimulus. The experiment was repeated twice with three different fish each. For gene expression analysis, primers sequences had been previously designed and tested (Thwaite et al., 2018) or were *de novo* designed selecting those genes reported to be upregulated within the interferon pathway of *O. mykiss* (Zou et al., 2005; Gao et al., 2009). A reference gene (elongation factor 1alpha (*ef1-α*)) and six selected genes of the innate immune response to viral infection (*vig1*, *mx*, *ccl4, il-1β, ifngr2,* and *ip10*) were used.

2.4. Functional characterization of VHSV-IFN^{NP} subunit vaccine in vivo

The functionality of the VHSV-IFN^{NP} vaccine was evaluated using two *in vivo* delivery models of rainbow trout: oral intubation and in-feed. In the first model, the intestinal absorption of the vaccine and its effect on the local immune response, as well as its biodistribution and systemic response in the head kidney and spleen, were assessed 24 h post-intubation. In addition, antibody titres and neutralizing capacity were determined 30 days after intubation (510 dd). Furthermore, in a small-scale trial of oral administration of the vaccine in feed, the humoral response to the VHSV vaccine was also evaluated by the production of specific antibody titres 35 days post-administration (455 dd).

2.4.1. Fish husbandry

For oral intubation, two groups of rainbow trout (*O. mykiss*) with average weights of 30 ± 2,5 g and 110 ± 23 g were maintained at the UAB fish facilities in tanks at 17 °C under a recirculating water system, light/dark regime of 12:12 h and fed twice a day at a rate of 2 % body weight with a commercial diet. For oral administration (vaccine in-feed), rainbow trout with average weights of ~2 g, were maintained in tanks at 13 °C with a dechlorinated water recirculation system, light/dark regime of 12:12 h at the animal facility and were carried out according to the criteria required by the Animal Welfare and the Research Ethics Committee of the Miguel Hernandez University (UMH), following the protocols established in Royal Decree 53/2013 and EU Directive 2010/63/EU. Fish were fed daily with a commercial diet at 3 % body

weight and were acclimatized for 2 weeks prior to the experiment. All experiments were performed following International Guiding Principles for Research Involving Animals (EU 2010/63) and the Ethics Committees of the Universitat Autònoma de Barcelona (UAB, CEEH) and of the Generalitat Valenciana (2019/VSC/PEA/0203).

2.4.2. Oral intubation of rainbow trout

Fluorescently labelled VHSV-IFN^{NP} and controls were administered by intubation to two groups of rainbow trout as described in section 2.4.4. Prior to administration, fish were anesthetized with MS-222 (50 mg/l) (Sigma-Aldrich). Atto labelled NPs were intubated into the fish in a volume of 200 μl (30 g fish) or 1 ml (110 g fish) using a 22G syringe with a thin silicon tube placed over the needle, which was introduced into the mouth, placing the tip end at the entrance of the oesophagus. Controls were fish intubated with 200 μl or 1 ml of PBS. Intubated fish were left to recover in aerated tanks before being returned to their tanks until sampling.

2.4.3. Fish feed manufacturing and in-feed oral vaccination

VHSV-IFN^{NP} vaccine and iRFP^{NP} control were included in the fish feed by means of a cold extrusion process developed by Life-Bioencapsulation SL (Spin-off from the University of Almería, Spain). Briefly, all the feed ingredients were mixed and ground with a hammer mill (UPZ 100, Hosokawa-Alpine, Augsburg, Germany) to 0.2 mm. The feed was extruded in a refrigerated P100 extruder (IFE, Italy) at lower temperature (40 °C), fitted with adequate die plates for manufacturing 0.7 mm sinking pellets. The pellets were dried at 30 °C using a drying chamber with forced-air circulation (Airfrio, Almería). A total of 500 g of feed (dry matter: 59 % crude protein, 15 % crude lipid, 11 % ash) were made with 1,1 g NPs/kg of feed. Once the feed was extruded, we tested whether the VHSV-IFN^{NP} could be isolated from the feed pellets and whether it remained intact. The antigen in the vaccine food was detected by Western Blot after de-lipidation and basic extraction process. Briefly, the pellets were first dried overnight at 50–60 °C. To remove lipids, the dried pellets were then incubated overnight with a mixture of chloroform and methanol in a 2:1 ratio. The chloroform phase was then removed by centrifugation, cold acetone was added, and the pellets were incubated at −20 °C for 20 min. The acetone supernatant was then removed by centrifugation and the pellets were allowed to dry at RT. After drying, they were incubated with 1 N NaOH at 40 °C overnight. Finally, feed samples were centrifuged to detect the His-tag by Western blot (Supplementary Fig. 1).

For in-feed oral vaccination, rainbow trout weighing 1–2 g ($n = 21$ animals per treatment) were acclimatized for 14 days prior to vaccine administration. The fish (Approximately 3 g) were vaccinated with a daily dose of 25 mg VHSV-IFN^{NP}/kg fish for 20 consecutive days or iRFP^{NP} at the same dosage, while the third group was fed commercial pellets.

2.4.4. Local response of gut leukocyte populations after oral administration of VHSV-IFN^{NP}

To assess the effect of the oral vaccine on the intestinal mucosal response, two studies of oral intubation in rainbow trout were performed. In the first study, fluorescently labelled VHSV-IFN^{NP} and iRFP^{NP} were administrated as a single dose of 30 mg/kg fish ($n = 4$) to 110 g trout, while in the second study, the NPs were administrated at 41.5 mg/kg fish/day for three consecutive days (total dose: 125 mg/kg fish; $n = 3$) to 30 g trout. For both trials, the control corresponds to fish intubated with PBS.

Considering previous intestinal uptake results at the same dose and intubation model (Supplementary Fig. 2), at 24 h post-administration, the hindgut was collected and processed for cytometry according to the procedures described above (Thwaite et al., 2018; Ji et al., 2018). Briefly, the hindgut was incubated in 1 ml of collagenase solution (collagenase Type IV) (Gibco) 1.5 mg/ml at RT under orbital shaker in the dark for 2 h. The tissue was disaggregated through a 100-μm cell strainer (Falcon, Corning), washing with PBS and cells were recovered by centrifugation at 400 x*g* for 15 min at 4 °C. After the last centrifugation, cells were resuspended in 300 μl of minimal medium (Leibovitz's L-15) for cell sorting (FACSJazz BD) from which leukocyte populations of interest were selected and recovered according to their size, complexity and fluorescence (FSC/SSC/FITC profile). These sorted cells were stored in TriReagent at −80 °C until RNA extraction. The genes included in the analysis were specific markers for T and B lymphocytes (*CD4, CD8α, IgM* and *IgT*), and APC cells (*MCSFR2a, CD11b, CD83, CD86, MHCI* and *MHCII*). Furthermore, the expression of key antiviral, pro-inflammatory and anti-inflammatory genes was also evaluated (Supplementary Table 1).

2.4.5. Bio-distribution of VHSV-IFN^{NP} vaccine administered by oral intubation

Vaccine biodistribution was assessed by two techniques: flow cytometry in fish intubated with a single dose of 30 mg/kg fish, and gene expression in fish intubated once with 30 mg/kg fish and for 3 consecutive days with a cumulative dose of 125 mg/kg fish. Fish were sacrificed 24 h after intubation with an overdose of MS-222 (200 mg/l). For uptake analysis, samples of the hindgut, head kidney (HK) and spleen were collected from each trout for cytometry analysis. HK and spleen were homogenized and incubated in DMEM + GlutaMAX, 10 % heat-inactivated FBS, and 100 μg/ml Primocin (Invitrogen) at 18 °C and 5 % CO_2. After 48 h, cells were trypsinized with 1 mg/ml Trypsin (Gibco) and then pelletized by centrifugation at 300 x*g* for 5 min at RT. Cells were resuspended in PBS for flow cytometry (FACSCalibur BD) and 10,000 events were analyzed. The hindgut was processed as described above for cytometry. For gene expression analysis, 100 mg of spleen and HK were collected and individually homogenized (Polytron PT 1600 D) in 1 ml of TriReagent according to the standard RNA extraction protocol. We assessed the expression of key antiviral, pro-inflammatory and anti-inflammatory genes, and the reference gene was elongation factor 1 alpha (*ef1-α*) (Supplementary Table 1).

2.4.6. Antigen specific antibody titers and neutralization assays

To determine the effect of the VHSV-IFN^{NP} oral vaccine on the adaptive immune system, indirect ELISAs were performed to detect the presence of specific antibodies against VHSV in the serum of 30 g fish immunized by intubation at 30 days post administration (total dose = 125 mg/kg fish; $n = 5$ per treatment) and in the serum of 3 g fish group at 35 days post vaccine feed administration (total dose = 500 mg/kg fish; $n = 8$ per treatment). The antibody titers were assessed as previously described (Puente-Marin et al., 2019). Briefly, a positive serum control from VHSV-challenged survivors was incorporated and five serum dilutions (1/30, 1/60, 1/120, 1/240 and 1/480) were tested. The primary mouse monoclonal was an anti-trout IgM antibody (1G7) (Puente-Marin et al., 2019) and the secondary antibody was a rabbit anti-mouse conjugated with peroxidase (RAM—Po) (Sigma-Aldrich). Detection was performed using 3,3′5,5′ tetramethylbenzine (TMB) substrate reagent set (BD Biosciences), and the absorbance was measured at 450 nm on a microplate reader (Bio Rad, iMARK).

In addition, a neutralization assay was performed to determine the neutralization capacity as previously described (Chico et al., 2009). Briefly, serum dilutions (from 1/25 to 1/1250) were incubated for 3 h at 14 °C with virus at a multiplicity of infection (MOI) of 3×10^{-2} in a serum:virus volume ratio of 1:10. A source of complement, 1 % volume of healthy trout serum, was then added and incubated for a further 30 min at 14 °C. Confluent EPC cells in 96-well plates were infected with the mixture (100 μl/well in triplicate). The controls used were as follows; healthy trout serum at 1 % volume, virus without additional serum and monoclonal antibody (MAb) 3FIA2, which is highly neutralizing to the VHSV glycoprotein (Fernandez-Alonso et al., 1998). Infected cells were incubated for 2 h at 14 °C, washed with PBS, and then incubation continued for 24 h in fresh culture medium (RPMI +2 % FBS). The VHSV infectivity was evaluated by an immunostaining focus assay, using the

primary antibody MAb 2C9 (Sanz et al., 1993) and the secondary antibody GAM-FITC (Sigma 4,600,042). Viral infection foci were detected on an INCell Analyzer 6000 imaging system (GE Healthcare Life Sciences).

2.5. RNA extraction, cDNA synthesis and qPCR

Total RNA was extracted using TriReagent (Sigma-Aldrich) according to manufacturer's instructions and quantified using nanodrop ND-1000 (Thermo Fisher Scientific). Then, cDNA was synthesized from 1 μg high quality total RNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR (qPCR) was performed at 60 °C in a CFX384 real-time PCR detection system (Bio-Rad) using iTaq Universal SYBR Green Supermix kit (Bio-Rad) following the manufacturer's instructions. Each PCR mixture consisted of 5 μl SYBR green supermix, 0.4 μM specific primers, 2 μl diluted cDNA and 2.6 μl water (Sigma-Aldrich) in a final volume of 10 μl. The dilution factor for all the genes tested was 1:10. Reference gene stability was calculated using RedFinder (Xie et al., 2012) and the best reference gene (*ef1α*) was selected. The primer sequences, amplification efficiencies, m value of standard curve and gene accession numbers have been listed in Supplementary Table 1. Data was analyzed using the Livak method (Livak and Schmittgen, 2001).

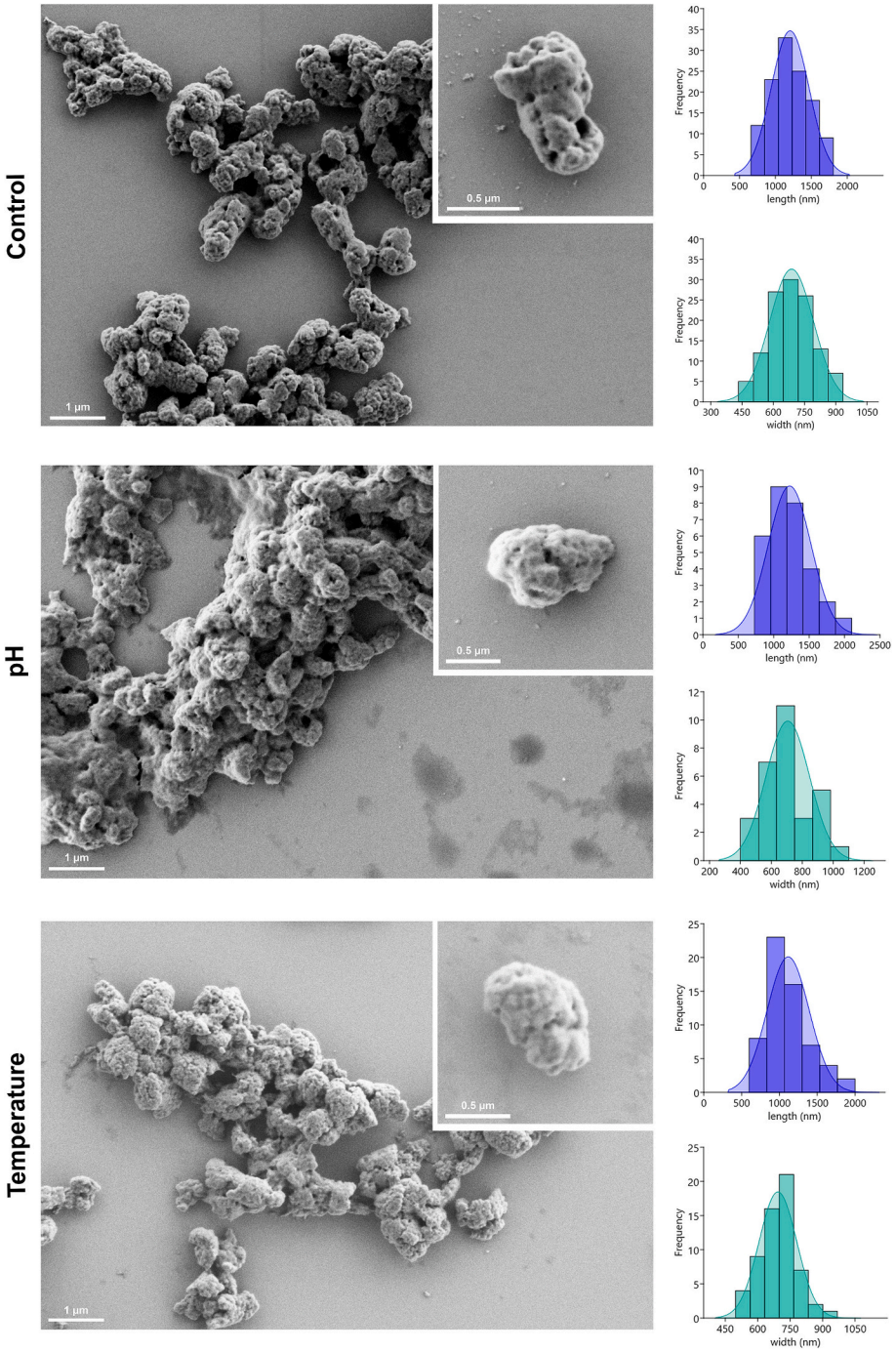

Fig. 1. Characterization of nanostructured VHSV-IFN^{NP} at low pH and high temperature. Field Emission Scanning Electron Microscopy images (FESEM) of VHSV-IFN^{NP} control, VHSV-IFN^{NP} incubated at pH 2 and VHSV-IFN^{NP} at 100 °C with the corresponding size distribution histograms (n = 60) for length (nm) and width (nm).

2.6. Statistical analysis

Statistical analysis and graphs were done with Prism 8.01 (GraphPad Prism). Comparisons of means for each experimental group *versus* control and between treatments (NPs) were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons with Brown-Forsythe, unpaired one-sided *t*-tests and Welch's correction for unequal variances or unpair nonparametric Mann-Whitney tests. Data are shown as mean ± standard deviation and ns: non-significant; $*p < 0.1$; $**p < 0.05$; $***p < 0.01$; $****p < 0.001$ was considered statistically significant.

3. Results and discussion

3.1. Characterization of the modular VHSV-IFN^{NP} subunit vaccine

Two different nanostructured recombinant viral proteins were expressed in *E. coli* and purified using non-chromatographic methods with an excellent yield: VHSV^{NP}, with a final yield of 11,7 mg/l and VHSV-IFN^{NP} with a final yield of 14,3 mg/l (Supplementary Fig. 3). Protein production yields are extremely protein-dependent although using the same plasmid. The VHSV-IFN^{NP} is expressed at higher levels compared to other viral antigens such as SVCV^{NP} and SVCV-IFN^{NP} (Rojas-Peña et al., 2022) or SAV^{NP} and IPNV-IFN^{NP} (unpublished data). The VHSV-IFN^{NP} vaccine production can be easily scaled up to meet industrial requirements using a bioreactor. Preliminary data using a 5 l bioreactor showed that we may easily increase the expression of VHSV-IFN^{NP} between 3 and 4 times reaching 40 mg/l (unpublished data).

The size and morphology of VHSV-IFN^{NP} were assessed by FESEM (Fig. 1). VHSV-IFN^{NP} nanoparticles had an ovoid shape and a porous surface with an average width and length of 712 ± 105 nm and 1205 ± 287 nm, respectively. The morphological features of nanostructured VHSV^{NP} were previously described in Thwaite et al. (Thwaite et al., 2018) with a rounder, smoother and smaller shape than VHSV-IFN^{NP}, features also observed in SVCV and SVCV-IFN^{NP} (Rojas-Peña et al., 2022).

VHSV-IFN^{NP} nanostructured protein was incubated at pH 2 and 100 °C to assess the resistance of the NPs to harsh conditions, like that of the gastrointestinal pH and the temperatures achieved during the aquafeed manufacture by extrusion. In both treatments, there were no significant changes in size and morphology (Fig. 1). The resistance of the NPs to high temperature and pressure was further tested when the vaccine was incorporated into fish feed by cold extrusion and the intact antigen was detected in manufactured vaccinal aquafeed extracts by Western blotting (Supplementary Fig. 1). We also demonstrated that feeding the fish with this diet, led to an immune response induced by the vaccine suggesting that the VHSV-IFN^{NP} can withstand the harsh gastrointestinal environment (see section 3.7). In contrast, other vaccines require encapsulation to protect them from the stomach conditions (Dhar et al., 2013; Mutoloki et al., 2015).

3.2. Uptake of subunit vaccine by RTgutGC and HKM cells

VHSV-IFN^{NP} was efficiently internalized by HKM and RTgutGC cells (Fig. 2). In dose–response experiments, both macrophages and enterocytes showed over 80 % of fluorescent-positive cells at all doses (Fig. 2a). The mean fluorescence intensity (MFI) increased with the dose, except for RTgutGC at 50 μg/ml where the intensity slightly decreased. In time course experiments, both HKM and RTgutGC cells showed similar percentages of uptake and MFI at 12 and 24 h after exposure to 10 μg/ml VHSV-IFN^{NP} (Fig. 2b). In previous studies, we observed a similar pattern of internalization of VHSV^{NP}, SVCV^{NP} and SVCV-IFN^{NP} in ZFL cells (Thwaite et al., 2018; Rojas-Peña et al., 2022), suggesting that these nanostructured proteins are readily internalized in different cell types.

To assess whether VHSV^{NP} and VHSV-IFN^{NP}, have similar internalization patterns, HKM and RTgutGC cells were exposed in parallel to 10 μg/ml of both NPs for 12 h. As shown in Fig. 2c, no differences in percentage of fluorescent cells were observed between both NPs. However, a higher MFI was observed in RTgutGC cells after VHSV-IFN^{NP} treatment. Different studies using RTgutGC cells, have reported variability in the percentage of uptake linked to the nature of the compound, the affinity for the cell receptor, and the size and surface charge of the particle (Alkie et al., 2019; Wang et al., 2019; Løkka et al., 2023).

VHSV-IFN^{NP} uptake was further confirmed by confocal microscopy both in HKM and RTgutGC cells (Fig. 2d). VHSV-IFN^{NP} were internalized and found in large aggregates within the cytosol. The 3D images (Fig. 2e) demonstrated the complete internalization of VHSV-IFN^{NP} in both cell lines.

3.3. Gene expression analysis in HKM after VHSV-IFN^{NP} treatment

Head kidney macrophages were stimulated with the VHSV-IFN^{NP} for 14 h at 20 μg/ml to test whether the VHSV-IFN^{NP} induce an antiviral response similar to that triggered by a viral infection (Fig. 3). In parallel, we treated the cells with iRFP^{NP} (20 μg/ml) an immunologically irrelevant protein, poly(I:C) (10 μg/ml) as a viral dsRNA mimic, and LPS as a bacterial infection mimic. As expected, we observed a strong antiviral response in HKM after poly(I:C) treatment, similar to that observed by Thwaite et al. (Thwaite et al., 2018), in both HKM and ZFL cells, inducing the overexpression of the whole antiviral panel of genes (Supplementary Table 1). As expected LPS induced a strong *il-1β* expression but not the expression of *ccl4* or *ip10* cytokines, whereas VHSV-IFN^{NP} was able to stimulate the gene expression of all anti-viral and pro-inflammatory genes with significant differences compared to the antigen alone (VHSV^{NP}). In addition, VHSV-IFN^{NP} was able to activate the expression of genes within the interferon signaling pathway, such as *ifgnr2* and *ip10* compared to iRFP^{NP} (Fig. 3). In a previous study using SVCV^{NP} and SVCV-IFN^{NP}, we reported how the addition of the recombinant IFN^{NP} module increased the antiviral response of SVCV^{NP} in ZFL cells (Rojas-Peña et al., 2022), which, together with the present study, would support the hypothesis that IFN would act as a adjuvant when bound to a viral antigen. Recombinant IFNs have been tested for their antiviral activity in different fish cell lines, demonstrating their potential as adjuvants for fish vaccines. A study by Kuo et al. (Kuo et al., 2016) evaluated the effects of different fish recombinant IFNs showing that salmon sIFN increased the expression of myxovirus resistance protein (*mx*), inhibit the NNV replication and reduce cytopathic effects (CPE) in NNV-infected grouper kidney (GK) cells. Morales-Lange et al. (Morales-Lange et al., 2021) studied the effects of IFNγ on the expression of cell surface markers in Atlantic salmon (*S. salar*) splenocytes and reported that they increased the gene expression and protein availability of CD80/86, CD83 and MHC II after stimulation with recombinant IFNγ. According to Bedekar et al. (Bedekar et al., 2018), *L. rohita* peripheral blood lymphocytes (PBMC) transfected with IFNγ displayed a significantly upregulation of *IFNγ, iNOS*, *mx*, and *il-1β* genes, just like cells treated with poly(I:C). This finding suggests that recombinant IFNγ may be just as effective as poly I:C. Sun et al. (Sun et al., 2011), investigated the antiviral activity of Atlantic salmon IFNγ in TO cells. They reported that IFNγ protected cells against infectious pancreatic necrosis virus (IPNV) and salmonid alphavirus 3 (SAV3), by reducing virus titres, inhibiting the synthesis of the viral protein VP3 and reducing viral nsP1 transcripts, respectively. The study also reported that IFNγ was a potent inducer of ISG15, the antiviral protein GBP, several interferon-regulated transcription factors (IRFs), *mx*, *vig1* and the chemokine *ip10*. The adjuvant activity of IFNs has also been tested in injectable vaccination models in fish. In a study with Atlantic salmon, the level of protection of a DNA vaccine against ISAV was evaluated by administering the antigen alone or by adding three different type 1 IFNs (IFNa1, IFNb or IFNc). The study demonstrated that the three IFN administered together with the viral antigen significantly enhanced the protection against ISAV-mediated mortality and stimulated an increase in specific IgM antibodies. Finally, in a previous study with SVCV^{NP} and SVCV-IFN^{NP}, we

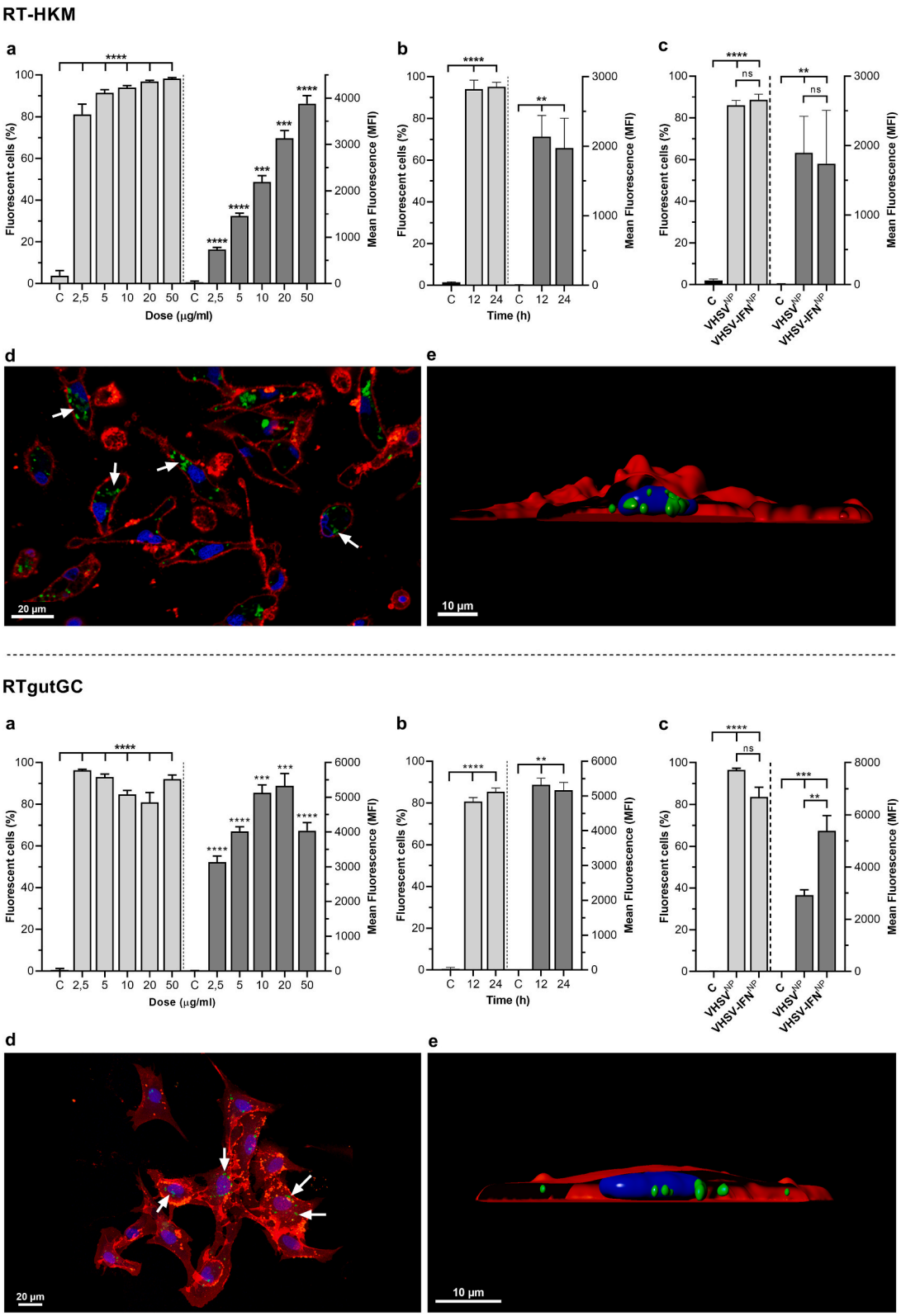

Fig. 2. Uptake of VHSV-IFN^{NP} by HKM and RTgutGC cells. (a) Dose response; (b) Time course; and (c) Comparative uptake between VHSV-IFN^{NPs} and VHSV^{NPs} evaluated by flow cytometry. For dose response assays 2,5 to 50 μg/ml of NPs were added to the cells and incubated for 12 h. For time course assays, 10 μg/ml of NPs were added to the cells and incubated for 12 and 24 h. For comparative analysis, 10 μg/ml of NPs were added to the cells and incubated for 12 h. Differences between means were analyzed by a one-way ANOVA with Tukey's multiple comparisons test, treatments *versus* control. Significance levels: ns, non-significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Uptake was evaluated by (d) Confocal microscopy at 20 μg/ml of NPs for 16 h; (e) digitalized image z-stacks, NPs (green, indicated by white arrows); cell membrane (red); nuclei (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

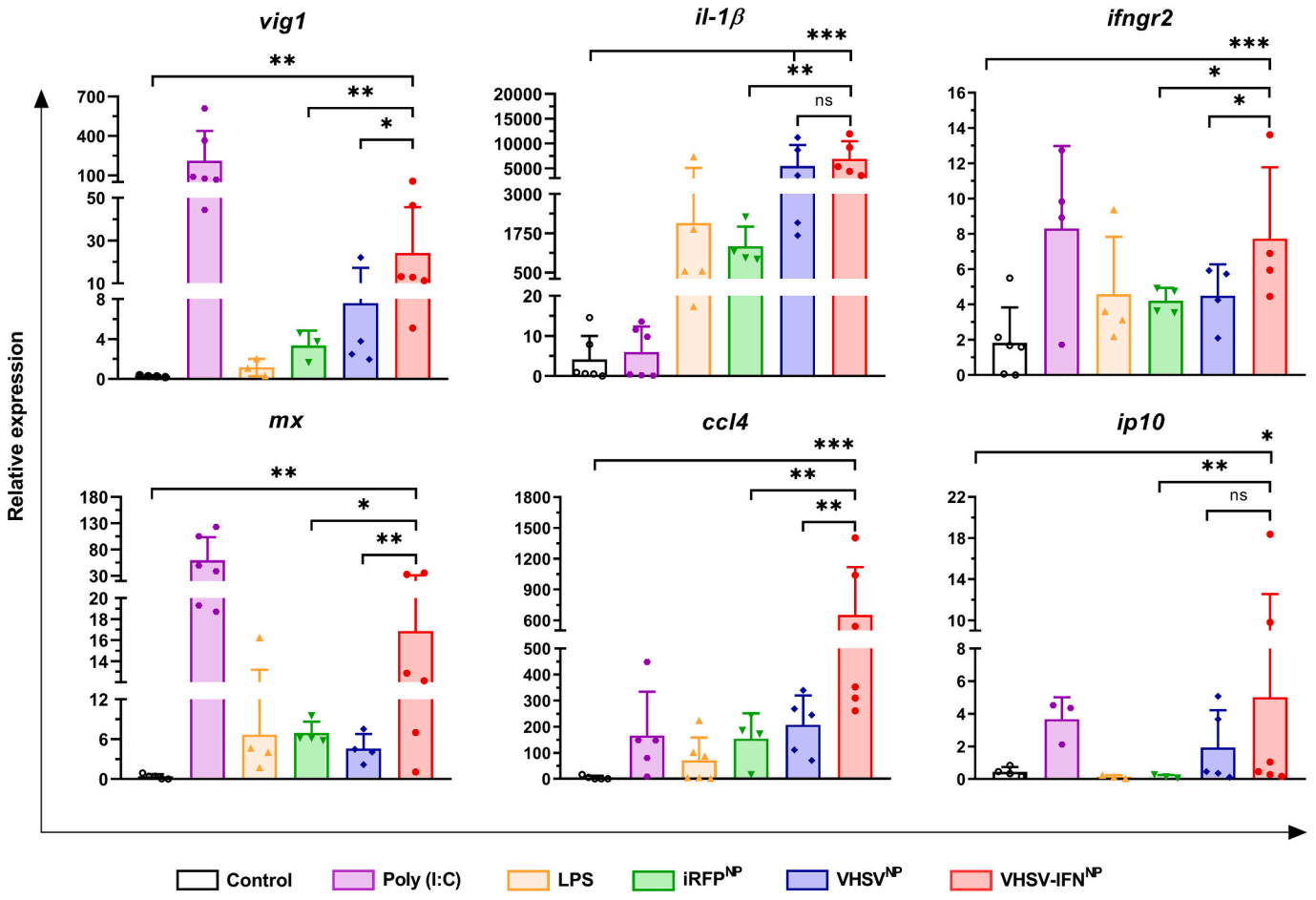

Fig. 3. Gene expression analysis of HKM treated with VHSV^{NP} and VHSV-IFN^{NP}. Cells were incubated as follow: unstimulated control cells (white), 10 μg/ml of poly (I:C) (purple) and LPS (orange), 20 μg/ml of VHSV^{NP} (blue) and VHSV-IFN^{NP} (red), and 20 μg/ml of iRFP^{NP} (green). Data are mean ± SD (n = 6). Statistical differences between treatments and controls were analyzed by unpaired one-sided *t*-tests with Welch's correction for unequal variances or unpair non-parametric Mann-Whitney test. Significance levels ns: non-significant; *$p < 0.1$; **$p < 0.05$; ***$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reported that the modular protein (antigen plus IFN) protected adult zebrafish against SVC virus infection resulting in significant higher survival rates than fish vaccinated only with the antigen.

3.4. Uptake of VHSV-IFN^{NP} vaccine administered by oral intubation

To assess intestinal absorption and vaccine biodistribution to immune-organs, VHSV^{NP} and VHSV-IFN^{NP} were administered by

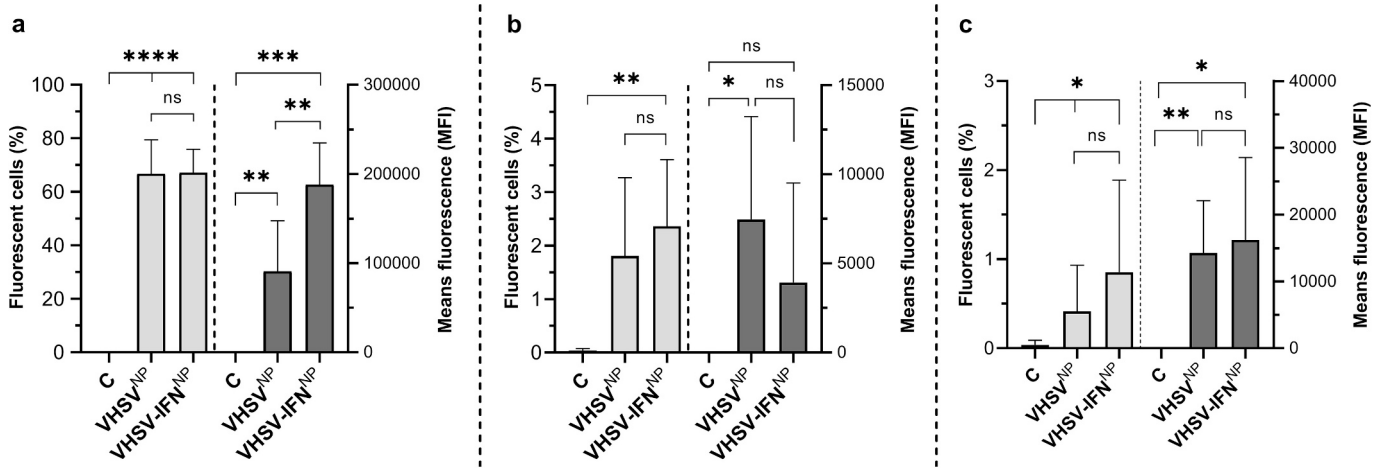

Fig. 4. Intestinal uptake and biodistribution of VHSV^{NP} and VHSV-IFN^{NP} by rainbow trout after intubation. Adult fish were intubated with 30 mg/Kg fish of fluorescently labelled NPs for 24 h ($n = 4$). (a) Hindgut, (b) spleen and (c) head kidney (HK) were sampled for cytometry analysis. Differences between means were analyzed by a one-way ANOVA with Tukey's multiple comparisons test. Significance levels ns: non-significant; *$p < 0.1$; **$p < 0.05$; ***$p < 0.01$; ****$p < 0.001$.

intubation at a dose of 30 mg/kg to 110 g trout and after 24 h samples of the hindgut, head kidney and spleen were collected and processed for cytometry. We performed preliminary studies to assess the pattern of NPs uptake using this intubation protocol, revealing that the hindgut showed the maximal uptake, both in percentage of fluorescent cells and fluorescence intensity, compared to the anterior section of the intestine (Supplementary Fig. 2). In Atlantic salmon the homeostatic mechanisms gradually increase from the foregut to the hindgut segments, with higher expression of immune-related genes, together with the appearance of smaller irregular intestinal folds from the foregut to the hindgut (Løkka et al., 2013; Løkka and Koppang, 2016). However, other studies have reported similar levels of expression of immune genes in the pyloric caecum, midgut and hindgut of cells isolated from rainbow trout (Attaya et al., 2018).

In this study, we detected over 60 % of uptake in the cells from the hindgut of fish intubated with both VHSV^{NP} and VHSV-IFN^{NP}. Besides, we observed a mean fluorescence intensity (MFI) that was twice as high in the case of the VHSV-IFN^{NP} (Fig. 4). Interestingly, we observed the same pattern in RTgutGC cells (Fig. 2c). RTgutGC cells are immortal cells from the hindgut of an adult female rainbow trout and has been described as having an epithelial-like morphology (Kawano, 2009; Wang et al., 2019), so we might expect that there would be common IFN receptors that would allow increased internalization of VHSV-IFN^{NP} in these cells. We also observed around 2 % of fluorescent positive cells with high MFI in the spleen and head kidney. However, no significant differences were observed between VHSV^{NP} and VHSV-IFN^{NP} (Fig. 4). The lower percentage of uptake in these organs compared to the intestine may be due to the short sampling time. The mechanism and timing of NPs uptake in the fish gut, as well as their biodistribution, remain unknown. This topic is discussed in section 3.6.

3.5. Characterization of the local immune response of gut leukocyte populations after VHSV-IFN^{NP} oral intubation

To confirm whether the uptake observed in the hindgut was true cellular internalization and to identify the cell types involved in the process, we characterize the hindgut cell populations by cell sorting and qPCR. The hindgut cell populations were isolated from 110 g trout and sorted 24 h after administration of VHSV-IFN^{NP} in two separate experiments: the first at a single dose of 30 mg/kg fish, and the second at a cumulative dose of 125 mg/kg fish after administration of the vaccine for three consecutive days. Based on the forward scatter (FSC) and the side scatter (SSC) analysis, we detected two large cell populations (Fig. 5a): epithelial cells (55 ± 23 %) and leukocytes (30 ± 16 %) similar to those reported in other studies (Attaya et al., 2018). At 30 mg NP/kg fish dose, we sorted three leukocyte populations according to SSC and fluorescence (FITC) tentatively identified as: myeloid-like cells (25 ± 13 %), lymphoid-like cells 1 (L1; 32 ± 1 %), and lymphoid-like cells 2 (L2; 21 ± 17 %) (Supplementary Fig. 4). Gene expression analysis showed that both lymphoid-like populations (L1 and L2) were functionally similar, with higher expression levels of *CD8α* and *MHCI* in fish stimulated with iRFP^{NP} and VHSV-IFN^{NP} than in the control group. Although L2 showed some expression of macrophage and dendritic cell markers, the expression was low, probably because this group may include some myeloid cells (Supplementary Fig. 4). Therefore, at 125 mg NP/kg fish dose, we decided to sort two populations of leukocytes, called lymphoid (26 ± 17 %) and myeloid (45 ± 33 %) (Fig. 5b), with the following characteristics: the lymphoid population showed high levels of expression of *CD8α* and *IgM*, T and B lymphocyte markers respectively, while the myeloid cells showed overexpression of APC markers (*MCSFR2a*, *MHCI* and *MHCII*). The importance of CD8^{+}T cells in intestinal mucosal immunity has been widely described, being the clearance of pathogen-infected epithelial cells their main function (Salinas, 2015). It has also been reported that CD8^{+} T cells make up the majority of the IELs (intraepithelial lymphocytes) population in the gut of teleosts, while CD4^{+} T cells would be in a lower proportion respect to other immune tissues (Salinas, 2015; Somamoto and Nakanishi, 2020; Lee et al., 2021). This is consistent with the results of the present study (Fig. 5C) and has also been reported in the intestine of sea bass (Picchietti et al., 2011) and leukocytes isolated from rainbow trout intestine (Attaya et al., 2018), where CD8^{+} T cells predominated over the CD4^{+} T cells subset. Although, a significant difference in the prevalence of IgT^{+} B cells relative to IgM^{+} B cells has been documented in the intestine of teleosts, indicating their important role in mucosal immunity (Zhang et al., 2010; Salinas et al., 2021), in our study we observed low levels of IgT^{+} B cell expression relative to IgM^{+} B cells (Fig. 5C). In line with our finding, relatively low levels of IgT transcripts were found in isolated trout GALT cells compared to the IgM expression levels (Attaya et al., 2018). This may reflect a lower number of IgT^{+} B cells in the sorted population. Nonetheless, the ability of IgM^{+} and IgT^{+} B cells to proliferate and secrete immunoglobulin in response to microbial stimulation has been shown to be essentially very similar (Zhang et al., 2010; Salinas et al., 2021). Interestingly, fish intubated with VHSV-IFN^{NP} and iRFP^{NP} showed higher overexpression of *IgM*, *MCSFR2a* and *MHCII* than the control group (Fig. 5c), which suggests that the NPs itself activate immune cells involved in the recognition of foreign agents. The production and purification of NPs in *E. coli* leave traces of contaminants (lipopolysaccharide, peptidoglycans and nucleic acids), which have been recognized as good adjuvants and immunomodulators (Ruyra et al., 2014). Regarding MHC molecules, Picchietti et al. (Picchietti et al., 2011) also found higher levels of *MHCI* expression compared to *MHCII* expression in the hindgut of sea bass, consistent with the overexpression of CD8^{+} T cells responsible for eliminating virus-infected cells. Consistent with this, we detected that VHSV-IFN^{NP} was able to stimulate the lymphoid population by increasing gene expression of antiviral and inflammatory genes with significant differences compared to iRFP^{NP} (Fig. 5c). Notably, this local antiviral response was not observed in the sorted populations from the hindgut of fish intubated with VHSV-IFN^{NP} at 30 mg/kg fish (Supplementary Fig. 4), suggesting a dose-response effect. It is noteworthy that no anti-inflammatory response (*il-10*) was observed with either dose, which preliminarily rules out oral tolerance to the vaccine, widely discussed in different reviews (Embregts and Forlenza, 2016; Løkka and Koppang, 2016; Lee et al., 2021).

3.6. VHSV-IFN^{NP} bio-distribution: Gene expression in HK and spleen after intubation

The head kidney and spleen isolated from 30 g trout in the VHSV^{NP} and VHSV-IFN^{NP} intubation experiments at doses of 30 mg/kg fish (section 3.4) and 125 mg/kg fish (section 3.5) were also evaluated to determine whether there would be an innate antiviral response as an indicator of vaccine biodistribution to these organs. At 30 mg/kg, we detected trends in the expression of some of the genes tested, but no relevant overexpression with either vaccine (Supplementary Fig. 5). However, at 125 mg/kg fish, although there were low levels of expression in the spleen, the VHSV-IFN^{NP} vaccine shows a clear trend towards stimulation of all tested genes, with significance in the antiviral genes in HK (Fig. 6). This may support the hypothesis that VHSV-IFN^{NP}, when administered at higher doses, was absorbed in the gut and biodistributed to other organs, eliciting an early antiviral response both locally (Fig. 5c) and systemically. However, we do not know the mechanism of NP biodistribution and the time required to activate the primary response in the intestine. It has been widely discussed that the mechanism of antigen transfer to the intraepithelial layer of the gut has not yet been elucidated, with divergences among different studies due to the high heterogeneity between different fish species and the lack of fish-specific biomarkers for differentiated GALT-associated leukocyte subpopulations, while agreeing that the transport of an exogenous antigen depends on its physical nature (Lee et al., 2021; Embregts and Forlenza, 2016; Muñoz-Atienza et al., 2021). For example, in a study with an alginate-encapsulated oral DNA vaccine (IPNV VP2 gene), they observed the same response in terms of time course and organ transcription

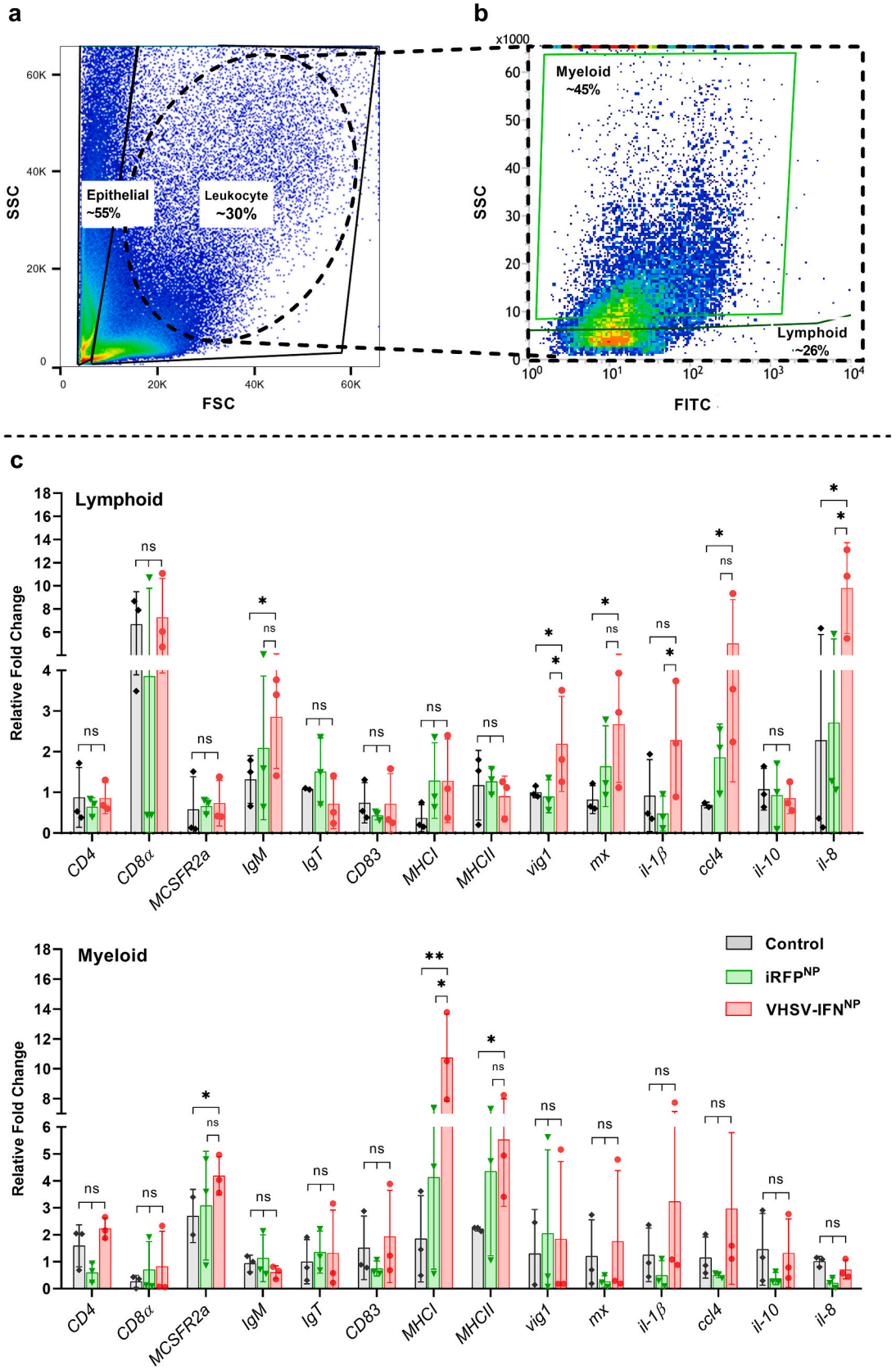

Fig. 5. Gene expression analysis of leukocyte marker genes. Cells were obtained from hindgut by cell sorting 24 h after the last administration with PBS (grey), VHSV-IFN^{NP} (red) and iRFP^{NP} (green), at 125 mg/Kg fish. (a) Intestinal cells FACS profile and (b) representative plot of 2 leukocytes population selected. (c) Expression of marker genes for T and B lymphocytes, and APC cells by qPCR. Bars are means ± SEM of three fish. Differences between means were analyzed by a one-way ANOVA with Tukey's multiple comparisons test or unpair non-parametric Mann-Whitney test. Significance levels ns: non-significant; *p < 0.1; **p < 0.05; ***p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

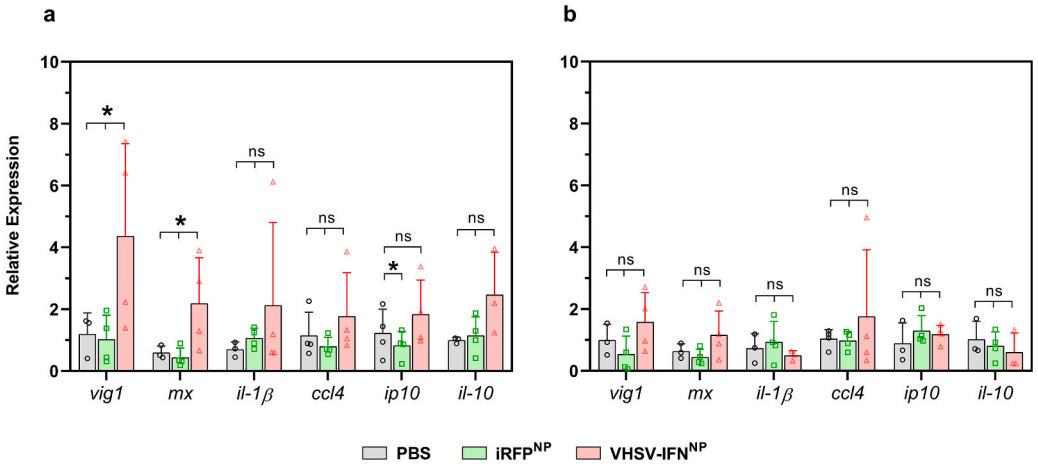

Fig. 6. Gene expression analysis of rainbow trout tissues induced with NPs by intubation. Adult rainbow trout (n = 4) were intubated for three consecutive days with a cumulative dose of 125 mg/kg fish of NPs. 24 h after the last intubation, (a) head kidney (HK) and (b) spleen were collected for qPCR analysis. Statistical differences between treatments were analyzed by unpaired one-sided t-tests with Welch's correction for unequal variances or unpair non-parametric Mann-Whitney test. Significance levels: ns, non-significant; *p < 0.1; **p < 0.05; ***p < 0.01.

profiles as an IPNV infection. Vaccination with VP2 induced a significant increase in the expression of immune-related genes (*stat1*, *ifn1*, *ifng*, *mx1*, *mx3*, *il-8*, *il-10*, *il-11*, *il-12b*, *tnf2*, *MHCI*, *IgM*) from day 3 to 10 post-vaccination, reaching a maximum on day 7 (Ballesteros et al., 2012). In a similar time-course pattern, it is possible that what we observed in HK 24 h after the last intubation of VHSV-IFN^{NP} at 125 mg/kg fish would be in an early phase of systemic antiviral activation, which may not yet be detectable in the spleen.

3.7. *Specific and neutralizing antibody titer elicited by VHSV-IFN^{NP} subunit vaccine*

To assess the level of protection provided by the VHSV-IFN^{NP} vaccine at 30 days post-intubation, the antibody titers were measured by indirect ELISA, and the inhibition of the VHS virus activity was assessed using a neutralization assay. Trout vaccinated with VHSV-IFN^{NP} induced a strong specific antibody response 30 days after intubation (510 degree-days), with significant differences in titers for the 1:30 sera dilution compared to iRFP^{NP}, PBS and non-immunized fish controls. Interestingly, the VHSV-IFN^{NP} vaccine, produced antibody levels comparable to the positive control corresponding to a surviving VHS-infected fish (Fig. 7a). Consistently, we observed that serum from fish vaccinated with VHSV-IFN^{NP} had the ability to reduce viral infection of cells compared to the iRFP^{NP} control, which had significantly higher viral loads relative to a highly neutralizing antibody (3FIA2) (Fig. 7b). However, there was some overlap between the vaccinated groups, showing a range in the neutralizing capacity (Fig. 7b). Similar results were previously observed in trout fry intraperitoneally injected (ip) with VHSV^{NP}, with higher antibody titres and neutralizing antibodies before and after a challenge with VHS virus compared to control fish (unpublished results). In addition, to compare the antibody titer of the VHSV-IFN^{NP} and the VHSV^{NP} vaccines, a trial was conducted in trout fry with

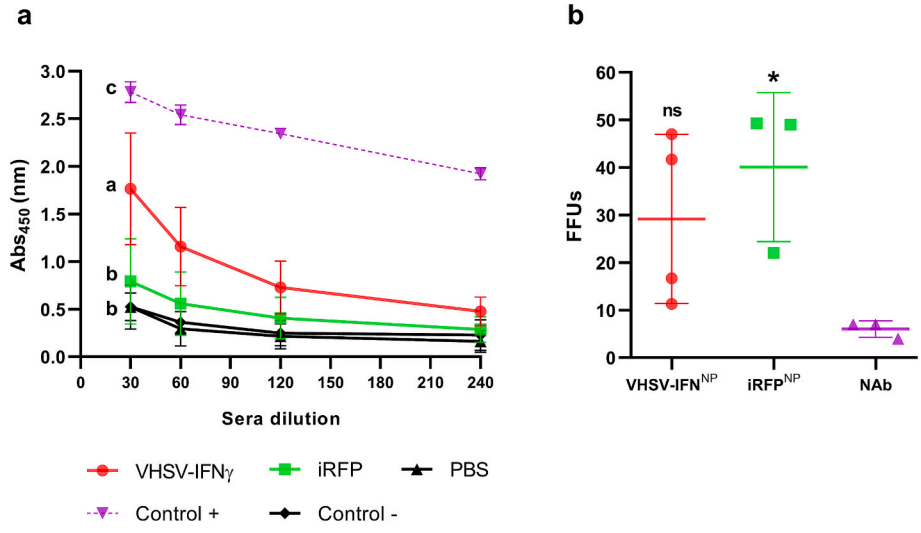

Fig. 7. IgM titers after VHS-IFN^{NP} vaccine administration by intubation. (a) Presence of specific anti-VHSV IgM and (b) Neutralizing capacity of sera diluted at 1:50 of rainbow trout immunized with VHSV-IFN^{NP}. Adult fish were intubated on 3 consecutive days with an accumulative dose of 125 mg/kg fish of NPs. Sera was obtained 30 days post immunization from: untreated control (black diamond), VHSV-IFN^{NP} vaccinated (red), iRFP^{NP} vaccinated (green), PBS control (black triangle), and VHSV infection survival fish positive control (purple). Data are mean ± SD (n = 5). (a) Differences between means were analyzed by a one-way ANOVA with Brown-Forsythe multiple comparisons test. Different letters indicate significant differences, and (b) differences between means were analyzed by non-parametric Mann-Whitney test with Dunn's multiple comparisons test. Significance levels: ns, non-significant; *p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both vaccines administered *via* ip. Similar antibody titres were obtained between the two vaccines being the titres two-fold higher in fish immunized with both VHSV^{NP} than fish injected with iRFP^{NP} (unpublished results). Interestingly in our study, we observed a similar pattern, with titers 2.3-fold higher in fish immunized with VHSV-IFN^{NP} *versus* fish immunized with the iRFP^{NP} (Fig. 7a). These results are very promising, considering that oral vaccines both in fish and humans induce weak or short-term protection compared to a parentally administered vaccine (Embregts and Forlenza, 2016).

To approach the oral administration of the VHSV-IFN^{NP} vaccine, we manufactured a vaccinal diet with VHSV-IFN^{NP} and a control diet with iRFP^{NP}, using a semi-industrial extrusion protocol. A laboratory-scale trial was carried out to evaluate the protective effect of the VHSV-IFN^{NP} vaccine in terms of antibody production and neutralizing ability at 35 days post administration (450 degree-days). The fish vaccinated with VHSV-IFN^{NP} increased the antibody titres compared to fish fed the control diet, but with no significant difference compared to iRFP^{NP} (Fig. 8a). However, we observed that fish fed the VHSV-IFN^{NP} vaccine were able to significantly reduce VHS-viral load compared to the iRFP^{NP} control at all dilutions tested (Fig. 8b). Overall, these results are positive considering the oral route of administration. In aquaculture, the parenteral route has been the most commonly used for vaccine administration. However, it often leads to adverse effects on the fish and incurs in high administration costs. Additionally, booster doses, whether through a new injection or oral administration, are typically required. In mammals, parenteral administration of antigens is known to induce weak mucosal responses, whereas administration of antigens to mucosal surfaces effectively induces both local humoral and systemic cell-mediated responses (Embregts and Forlenza, 2016; Neutra and Kozlowski, 2006). Despite the advantages of mucosal vaccines, there are only a few vaccines (immersion and oral) on the market. The lack of protective correlates, the inability to optimize protective doses, the possibility of oral tolerance, the potential for gastric denaturation of oral vaccines and the ability of antigens to cross mucosal barriers to access antigen-presenting cells (APCs) are just some of the difficulties that have hampered their development (Ma et al., 2019; Embregts and Forlenza, 2016; Adams, 2019). Despite these obstacles, some antiviral oral vaccines have been shown to overcome mucosal tolerance and induce effective local and systemic immune responses. Some examples include an oral vaccine against ISA which contains an encapsulated recombinant protein (Caruffo et al., 2016), and shows a relative percent survival (RPS) of 64.3 %. This vaccine also induces the production of specific ISAV antibodies from 330 degree-days (dd) post administration. This is like our study, where fish immunized with VHSV-IFN^{NP} vaccine showed antibody titers two-fold higher at 360 dd after intubation and 450 dd after oral administration. In another study, a recombinant NNV vaccine (rNNV) produced in *E. coli* (Gonzalez-Silvera et al., 2019), and delivered through food pellets containing the whole recombinant bacteria, showed a significant increase in NNV-specific IgM 30 days after vaccination, although the intraperitoneal route induced higher antibody levels. Both routes resulted in 100 % RPS after the NNV challenge. Additionally, oral vaccination of trouts with *L. lactis* expressing the G gene of VHSV showed high levels of specific antibodies between day 14 and day 60 post-administration. At the highest dose, the RPS was 78 % (Naderi-Samani et al., 2020). It is important to note that the main differences between these studies and our research are that they used either encapsulation or boosters for administration.

4. Conclusions

Here we present a recombinant subunit vaccine against VHSV that incorporates an IFNγ module as an adjuvant. This vaccine significantly enhanced the immune response *in vitro*, increases the intestinal uptake of the NPs, and activates both local and systemic antiviral responses in a dose-dependent manner following oral intubation. Moreover, this vaccine triggers an adaptive immune response leading to the production of specific and neutralizing antibodies when administered either by intubation or when incorporated into fish feed, highlighting its potential for oral delivery. Furthermore, this vaccine is highly stable (does not require critical transport and storage conditions), does not need encapsulation and is easily scalable for production systems.

Authors contributions

PA, MR, MG and BO performed the production and purification of NPs; PA and MR performed the experiments; PA, MR and NR designed experiments and analyzed data; PA and NR wrote the manuscript draft; NR conceived ideas and NR, LP, VC, FA and MO oversaw the research. All authors were involved in discussions and contributed to the final writing of the manuscript.

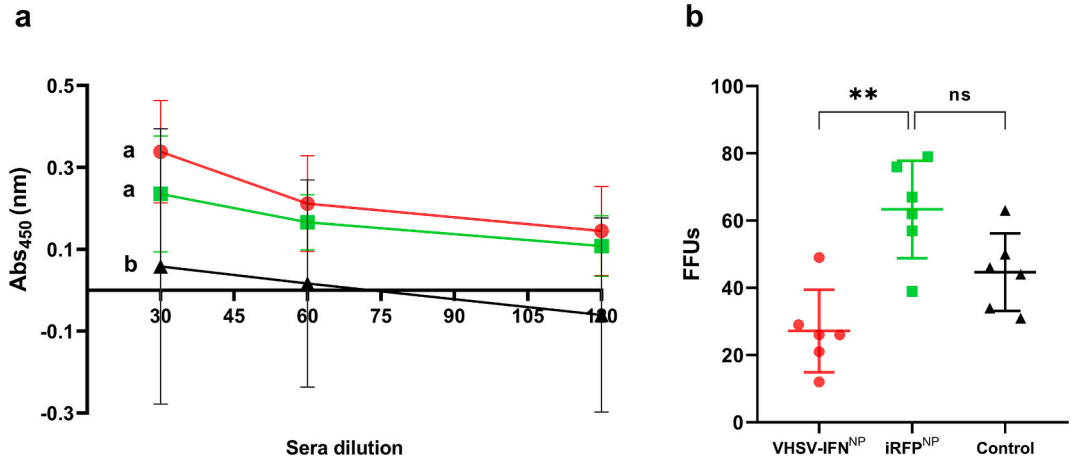

Fig. 8. Antibody levels provided by VHS-IFN^{NP} vaccine administered *via* oral administration. (a) Presence of specific anti-VHSV IgM and (b) Neutralizing activity of sera diluted at 1:50 of rainbow trout immunized with VHSV-IFN^{NP}. Trout were feed for 20 days with iRFP^{NP} (green) and VHSV-IFN^{NP} (red) vaccines in-feed, and commercial feed (black). After 35 days, fish sera were collected, and the presence of anti-VHSV-G protein specific antibodies was analyzed by ELISA using concentrated VHSV. Bars represent the average values and standard deviations ($n = 8$). (a) Differences between means were analyzed by a one-way ANOVA with Brown-Forsythe multiple comparisons test. Different letters indicating significant differences, and (b) differences between means were analyzed by non-parametric Mann-Whitney test with Dunn's multiple comparisons test. Significance levels ns: non-significant; *$p < 0.05$; **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ethics statement

All methods were performed in accordance with the Spanish and European regulations (RD53/2013 and EU Directive 2010/63/EU) for the protection of animals used for research experimentation and other scientific purposes. Rainbow trout procedures were conducted under Generalitat Valenciana permit 2019/VSC/PEA/0203.

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CRediT authorship contribution statement

Patricia Aceituno: Writing – original draft, Methodology, Investigation, Conceptualization. **Mauricio Rojas-Peña:** Software, Methodology, Formal analysis, Data curation, Conceptualization. **Maria Salvador-Mira:** Formal analysis. **Marlid Garcia-Ordoñez:** Formal analysis. **Verónica Chico:** Formal analysis. **Borja Ordóñez-Grande:** Formal analysis. **Francisco Javier Alarcón:** Resources. **Luis Perez:** Validation, Supervision. **María del Mar Ortega-Villaizán:** Validation, Supervision. **Nerea Roher:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that the research was performed without any conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742794.

Data availability

Data will be made available on request.

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