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Functional nutrition modulates the early immune response against viral haemorrhagic septicaemia virus (VHSV) in rainbow trout

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ABSTRACT

Although viruses represent a major threat for cultured fish worldwide, the commercialization of vaccines capable of providing effective and long-lasting protection is still lacking for most of these viral diseases. In this situation, the use of supplemented diets could be a suitable strategy to increase the immune status of the fish and reduce the impact of viral pathogens. Among possible immunostimulants that could be included in these functional feeds, some studies have previously shown that certain β-glucans can significantly increase certain immune parameters of fish and reduce the impact of viral diseases. However, the mechanisms through which β-glucans exert their activity have not been fully elucidated yet. In the current study, we have studied the immune response of different tissues to viral haemorrhagic septicaemia virus (VHSV) in rainbow trout fed with a non-supplemented control diet as well as in fish fed a commercial functional aquafeed (Protec™, Skretting) containing β-glucans, vitamin C, vitamin E and zinc. For this, after 30 days of feeding the fish with one of the two diets, they were subsequently infected with VHSV by bath or mock-infected. After 2 or 6 days post-infection, fish were sacrificed and the levels of transcription of different immune genes such as IgM, IgT, IgD, Mx, interferon γ (IFN γ) and perforin studied in different tissues (kidney, gut and gills). Additionally, the levels of natural IgMs in serum were also determined. Our results demonstrate that fish fed the functional diet were capable of mounting an increased IgM, IgT, IgD and Mx transcriptional response to the virus. Additionally, these fish also showed increased levels of natural IgMs in serum. These results reveal a previously undescribed effect of functional diets on fish Ig production and point to Protec™ as an adequate diet to be incorporated in holistic programs aimed at mitigating the effect of viral diseases.

1. Introduction

One of the most relevant factors that limits the required expansion of the aquaculture industry is the impact of infectious diseases. As a consequence of intensive rearing conditions in fish farms, with high levels of organic material and low oxygen concentration in the water, cultured fish commonly experience a chronic stress condition that favors the appearance of diseases and increases their susceptibility to pathogens. Thus, infectious diseases severely affect the aquaculture production worldwide provoking major economic losses each year. These losses are not a consequence of the direct fish deaths but are also due to the impact that pathogens have on fish growth, production costs or reproduction cycles. Fish can be infected by different types of pathogens including virus, bacteria, fungi or unicellular or multicellular parasites. Among them, viral infections represent a major threat for cultured fish because the diseases they provoke usually elicit high mortality rates and there are no antivirals authorized for use in aquaculture [1].

Viral haemorrhagic septicaemia virus (VHSV), a member of *Rhabdoviridae* family included in the genus *Novirhabdovirus* [2] is responsible for one of the most devastating diseases of aquacultured fish. VHSV was first isolated in rainbow trout (*Oncorhynchus mykiss*) in 1965 in Denmark [3], however, it now has been now reported to infect more than 80 different fish species from fresh and saltwater [4]. Traditionally, it was thought that VHSV, as other closely related fish rhabdoviruses such as infectious haematopoietic necrosis virus (IHNV) entered the host through the gills, but in 2006, through the use of a luciferase-expressing recombinant rhabdovirus, it was established that these viruses also enter the host through the fin bases, being this the most important early replication site [5]. The most common symptoms of

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VHS include general haemorrhages, exophthalmia, anaemia and distended abdomen as a consequence of intraperitoneal cavity oedema [6].

Vaccination is, from all points of view, the most adequate way to prevent the appearance of viral diseases. However, few antiviral vaccines are commercially available for use in aquaculture and, in general, vaccines against fish viruses are not capable of generating an effective long-lasting protection in the field. In the case of VHSV and other related rhabdoviruses, DNA vaccination has been shown to generate a robust and long-lasting specific response when administered intramuscularly [7,8]. These DNA vaccines consist of a eukaryotic expression plasmid that encodes the glycoprotein of fish rhabdoviruses under the control of the cytomegalovirus (CMV) promoter. Despite its efficacy, this VHSV DNA vaccine has never been approved by the European authorities due to safety concerns, and thus far, only an IHNV DNA vaccine has been approved for use in Canada (Novartis).

In this situation, an alternative strategy to increase the natural resistance of fish to viral infections could be the introduction of immunostimulants in the diet. Immunostimulants are defined as natural or chemical compounds that have the capacity to activate or modulate non-specific immune responses, thus improving the general immune status of the fish and consequently increasing its natural resistance to pathogens [9]. Numerous studies over the years have confirmed that the use of immunostimulants such us plant or algae extracts, vitamins or oligoelements have the capacity to enhance the innate immune system in different fish species (reviewed in Refs. [9,10]). Among the most frequently used immunostimulants in aquaculture are β-1,3/1,6-glucans [11,12]. These compounds are glucose polymers that make up the cellular wall of plants, fungi and some bacteria [12]. β-glucans have been administered orally, by peritoneal injection or by bath, alone or in combination with vaccines (as adjuvants), and in many cases these compounds generated a positive effect in the immune system [13–15,42,43]. In addition, fish fed with diets rich in β-glucans have demonstrated increased resistance against bacterial pathogens such as *Vibrio harveyi* [16] or *Aeromonas hydrophila* [17] as well as against some viruses [18,19]. Thus, for example, Pacific herring fed β-glucan-supplemented diets showed a 50–80% increased protection when challenged with VHSV [19]. Despite this, the mechanisms through which supplemented diets exert their activity against viral infections in fish are still not well documented.

Additional supplements such as vitamins or zinc have also been proved to increase the natural defences to pathogens in aquaculture. Thus, for example, vitamin C has been shown to up-regulate several immune functions of rainbow trout leucocytes [20]. Regarding the effect of vitamins on the susceptibility to viral infections, Wahli and colleagues demonstrated that fish fed with a diet supplemented with a combination of both vitamin C and E at high doses had lower susceptibility to VHSV than fish fed with diets supplemented with lower vitamin doses or with either of the vitamins alone [21].

In the current work, we have studied the transcriptional response of different tissues at early time points after VHSV infection comparing the responses of rainbow trout fed with the functional aquafeed Protec™ (Skretting) to that of fish fed with a control non-supplemented feed. Protec™ is a commercial diet supplemented with a combination of glucans, vitamin C, vitamin E and zinc. For this, rainbow trout were fed each of the two diets for 30 days. Thereafter, fish were infected with VHSV through bath exposure and the levels of transcription of different immune genes determined in infected and mock-infected controls at days 2 and 6 post-infection. Additionally, the presence of natural IgMs in serum were also studied in sampled fish. These studies contribute to increase our knowledge on how functional diets exert their antiviral effects and consolidate Protec™ as a suitable diet to diminish the impact of viral diseases.

2. Materials and Methods

2.1. Experimental fish

Rainbow trout (*Oncorhynchus mykiss*) of ~25 g were obtained from the *Centro de Acuicultura El Molino* (Madrid, Spain). Fish were maintained at animal facilities of the Animal Health Research Center of the *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (CISA-INIA) in a recirculating water system at 14 °C, with 12:12 h light/dark photoperiod. Prior to any experimental procedure, animals were acclimatized to laboratory conditions for at least 2 weeks. During this period, fish were fed twice a day with a commercial standard diet (Skretting Spain, S.A., Burgos, Spain). All of the experiments described comply with the Guidelines of the European Union Council (1010/63/EU) for the use of laboratory animals and have been approved by INIA Ethics Committee (ORCEEA 2016-021).

2.2. Experimental design

After the acclimation period, rainbow trout were divided in two groups of 16 fish each. One group was fed for 30 days with a functional diet (Protec™, Skretting), a commercial functional feed containing a combination of β-glucans, vitamin C, vitamin E and zinc, whereas the other was fed with a control diet containing the same ingredients without the supplementation of immunostimulants. In both cases, fish were fed 2% of their body weight per day. Thereafter, both groups were divided in two smaller groups (n = 8). In each case, one group was infected by bath with VHSV 3592 (5×10^6 $TCID_{50}$/ml) or mock-infected as previously described [22]. During the infection period, fish were fed the corresponding diet previously used in the 30 day feeding period. After 2 or 6 days post-infection, four trout in each group were killed by a benzocaine (Sigma) overdose. Blood was then extracted from the caudal vein and kidney, gut and gills removed and placed in TRI Reagent Solution (Invitrogen).

2.3. Transcriptional analysis

Total RNA was isolated from kidney, gut and gills using TRI Reagent Solution following the manufacturer's instructions. The RNA pellet was washed with 75% ethanol, dissolved in RNAse-free water and stored at −80 °C until use. One μg of RNA was treated with DNAse I (Thermo Scientific) to remove any genomic DNA traces that might interfere with the PCR reactions and then used to obtain cDNA using the Superscript II reverse transcriptase (Life Technologies) following a protocol previously described [23]. The cDNA was diluted in a 1:10 proportion with RNAse-free water and stored at −20 °C.

To evaluate levels of IgM, IgT, IgD, Mx, IFNγ and perforin transcription, real time PCRs were performed in a LightCycler 96 System instrument (Roche) using FastStart essential DNA green master (Roche) and specific primers previously optimized (Table 1). Each sample was measured under the following conditions: 10 min at 95 °C, followed by 40 amplification cycles (10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C). The expression of individual genes was normalized to relative expression of trout elongation factor 1α (EF-1α) and the expression levels were calculated using the $2^{-\Delta Ct}$ method, where ΔCt is determined by subtracting the EF-1α value from Ct of the targeted gene as previously described [23]. EF-1α was selected as reference gene according to the MIQE guidelines [24]. A statistical analysis determined there were no differences between the means of the expression of EF-1α among experimental groups. Negative controls with no template and *minus* reverse transcriptase controls (-RT) were also included.

2.4. Determination of IgM titers by ELISA

Serum samples were obtained after blood clotting at room temperature (RT) for 1–2 h followed by incubation overnight at 4 °C. The

Table 1
Primers used for real time PCR analysis in this study.

Gene	Forward	Reverse
EF1α	GATCCAGAAGGAGGTCACCA	TTACGTTCGACCTTCCATCC
Total IgM	TGCGTGTTTGAGAACAAAGC	GACGGCTCGATGATCGTAAT
IgD	AGCTACATGGGAGTCAGTCAACT	CTTCGATCCTACCTCCAGTTCCT
IgT	AACATCACCTGGCACATCAA	TTCAGGTTGCCCTTTGATTC
Mx	AGCTCAAACGCCTGATGAAG	ACCCCACTGAAACACACCTG
IFNγ	GAAGGCTCTGTCCGAGTTCA	TGTGTGATTTGAGCCTCTGG
Perforin	GGAACGACGACCTGTTAGGA	TCATAGGGGAGGGCACATAG
VHSV-G	AAGGATCACGAGTACCCGTTCTTC	CCCAATAGACTCCCTGCCAATG

levels of total IgM were then determined following a slight modification of the protocol described by Abos et al. [25]. Briefly, 96-well ELISA plates were coated overnight with 100 μl of anti-trout IgM monoclonal antibody (1.14, 2 μg/ml). Wells were then blocked with 100 μl of 1% BSA in 1% Tween-20 PBS for 1 h at RT. Plates were washed with PBS-1% Tween-20 and serum samples diluted 1:10, 1:100 and 1:1000 in PBS-1% BSA added in duplicate to the wells (100 μl per well). Samples were incubated 1 h at RT and washed again in PBS-1% Tween-20. Then, 100 μl of biotinylated anti-trout IgM monoclonal antibody (4C10, 1 μg/ml) diluted in blocking buffer were added to the wells and samples incubated for 1 h at RT. After washing three times with PBS-1% Tween-20, plates were incubated with 50 μl of Streptavidin-HRP (500 ng/ml in PBS-1% BSA) for 1 h at RT. Wells were washed again and 50 μl of *o*-Phenylenediamine dihydrochloride (OPD, Sigma) (1 μg/ml) added as substrate. Absorbance at OD_{490} was measured in a FLUO Star Omega Microplate Reader, and mean and standard deviation for each fish calculated.

2.5. Statistics

Data handling, analyses and graphic representation were performed using Microsoft Office Excel 2010. Statistical analyses were performed using a two-tailed Student's *t*-test, and mean values were considered statistically different when $P < 0.05$.

3. Results

3.1. IgM transcription in kidney, gut and gills of fish fed with Protec™ and challenged with VHSV

We first analysed the levels of transcription of IgM in the tissues obtained from the sampled fish. In the kidney, in mock-infected animals sampled 2 days post-infection, IgM transcription levels were significantly higher in fish fed with the functional diet than in fish fed with the control diet (Fig. 1A). We could consider these basal transcription levels. Upon VHSV infection, only fish fed with Protec™ were able to significantly increase IgM mRNA levels in the kidney in response to the virus. At day 6 post-infection, again IgM transcription levels were significantly higher in fish fed with the supplemented diet than in fish fed with the control diet in the absence of VHSV infection (Fig. 1A). In this case, VHSV increased IgM mRNA levels both in fish fed the control diet and in fish fed with Protec™, but the levels of IgM transcription reached significantly higher levels in the latter (Fig. 1A).

In the gut, mock-infected fish that were fed Protec™ had IgM transcription levels significantly higher than fish fed the control diet, but only in fish sampled at day 6 post-infection (Fig. 1B). After 2 days of infection with VHSV, we observed that the levels of IgM transcription were significantly induced in comparison to mock-challenged fish, but again the IgM mRNA levels reached in fish fed with the functional feed were significantly higher than those reached in fish fed the control diet (Fig. 1B). A similar response was observed at day 6 post-infection, although at this point, only fish fed with Protec™ responded significantly to the virus infection (Fig. 1B).

In gills, mock-infected animals fed with the functional diet had IgM transcription levels significantly higher than those detected in fish fed with the control diet (Fig. 1C). This was consistently observed both in fish sampled at day 2 or at day 6 (Fig. 1C). In this case, VHSV did not significantly affect the levels of IgM transcription in fish fed the control diet, while on the other hand, it provoked a significant decrease of IgM transcription levels at day 2 and a significant increase at day 6 in fish fed Protec™ (Fig. 1C).

3.2. IgT transcription in kidney, gut and gills of fish fed with Protec™ and challenged with VHSV

Concerning the levels of IgT transcription, we found that VHSV significantly increased the levels of IgT transcription in fish fed with the control diet at day 2, while it was not until day 6 post-infection that the virus induced IgT mRNA levels in fish fed with Protec™ (Fig. 2A). At this point, IgT mRNA levels were no longer up-regulated in response to the virus in fish fed the control diet.

In gut, we found that both in fish sampled at day 2 and at day 6, basal IgT transcription levels (found in mock-infected fish) were significantly higher in fish fed the functional feed than in fish fed the control diet (Fig. 2B). At this point, the virus had no significant effect on the IgT mRNA levels in any of the groups.

In gills, no significant differences in the basal levels of IgT transcription were observed at any of the days sampled (Fig. 2C). Interestingly, at day 2 post-infection, the levels of IgT transcription decreased in response to VHSV both in fish fed the control diet and in fish fed Protec™ (Fig. 2C). This decrease was significantly higher in fish fed Protec™ than in fish fed the control diet (Fig. 2C) and was no longer visible at day 6.

3.3. IgD transcription in kidney, gut and gills of fish fed with Protec™ and challenged with VHSV

We also analysed the levels of transcription of IgD in kidney, gut and gills of experimental fish (Fig. 3A). No significant differences in basal IgD mRNA levels were observed in the kidney at neither of the time points sampled (Fig. 3A). At day 2 post-infection, VHSV induced a significant increase in IgD transcription in Protec™-fed animals that was not visualized in fish fed the control diet (Fig. 3A). This effect was not maintained at day 6 (Fig. 3).

We found higher basal IgD transcription levels in the gut of fish fed the functional diet than fish fed with the control diet, when sampled at day 2 (Fig. 3B). Interestingly, VHSV significantly increased the levels of IgD transcription in all experimental groups both at day 2 and 6 post-infection (Fig. 3B), however, the IgD mRNA levels reached in fish fed Protec™ were significantly higher than those fed the control diet (Fig. 3B).

In gills, as in the gut, higher basal IgD transcription levels were found in fish fed Protec™ when compared to fish fed with the control diet, when sampled at day 2 (Fig. 3C). This difference was no longer maintained in fish sampled at day 6 (Fig. 3C). At day 2 post-infection, VHSV had no effect in IgD expression levels, but at day 6 post-infection,

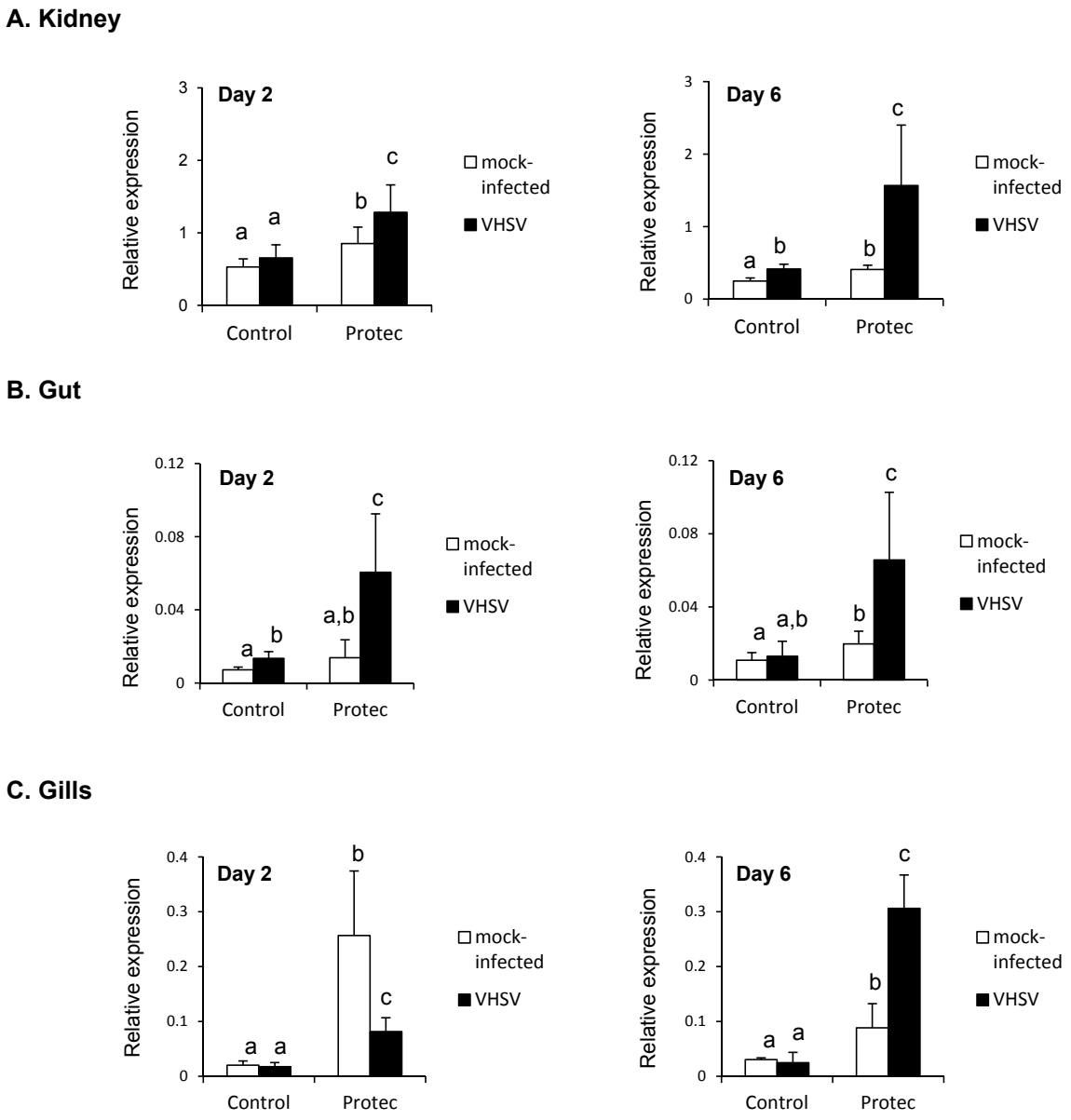

Fig. 1. Transcription levels of IgM in kidney, gut and gill of rainbow trout fed with Protec™ and challenged with VHSV. Rainbow trout were fed for 30 days with Protec™ or a control diet. Thereafter, both groups were divided in two and either infected by bath with VHSV (5×10^6 [43] $TCID_{50}$/ml) or mock-infected. At days 2 and 6 post-infection, fish were sacrificed and the levels of transcription of IgM in kidney, gut and gills determined by real-time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF-1α ± SD (n = 4). Different letters denote statistically differences among groups ($P < 0.05$).

only fish fed the supplemented feed were capable of increasing IgD transcription levels in response to the virus.

3.4. Mx transcription in kidney, gut and gills of fish fed with Protec™ and challenged with VHSV

We studied the levels of transcription of Mx as an indicator of type I IFN production as performed in the past [26]. In the case of Mx, no significant differences were observed among experimental groups at day 2 post-infection in the kidney (Fig. 4A). At day 6 post-infection, only fish fed with Protec™ were capable of significantly up-regulating Mx mRNA levels in the kidney in response to the virus (Fig. 4A).

In the gut, VHSV significantly up-regulated Mx transcription both after 2 and after 6 days post-infection (Fig. 4B). Although no significant differences were observed among Mx transcription levels from the different VHSV-challenged groups, the basal Mx mRNA levels were higher in fish fed the supplemented feed than in fish fed the control diet, when sampled at day 6 (Fig. 4B).

In gills, basal Mx transcription levels were higher in fish fed Protec™ than in fish fed the control diet when sampled at day 2 (Fig. 4C). This difference was no longer visible when fish were sampled at day 6 (Fig. 4C). At this later point, VHSV significantly induced Mx transcription both at similar levels in both experimental groups (Fig. 4C).

3.5. IFNγ transcription in kidney, gut and gills of fish fed with Protec™ and challenged with VHSV

We also determined how type II IFN (IFNγ) was affected by the different feeding regimes and in response to viral infection. In the kidney, no significant differences were observed among the levels of IFNγ transcription among experimental groups sampled at day 2 (Fig. 5A). At day 6 post-infection, VHSV significantly increased IFNγ

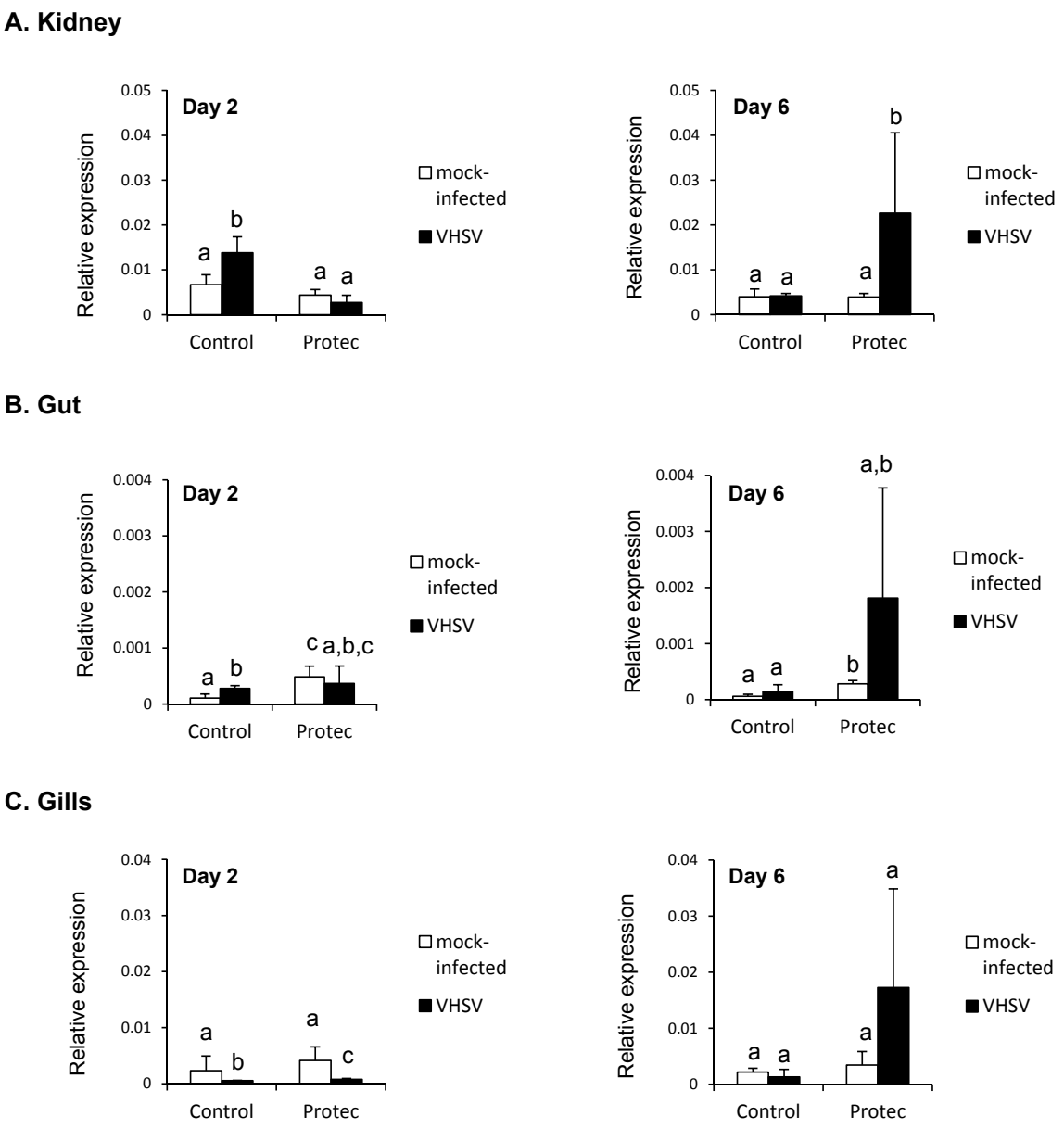

Fig. 2. Transcription levels of IgT in kidney, gut and gill of rainbow trout fed with Protec™ and challenged with VHSV. Rainbow trout were fed and challenged as described in the legend of Fig. 1. At days 2 and 6 post-infection, fish were sacrificed and the levels of transcription of IgT in kidney, gut and gills determined by real-time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF-1α ± SD (n = 4). Different letters denote statistically differences among groups ($P < 0.05$).

mRNA levels both in control and Protec™- fed fish, with no differences between them (Fig. 5A).

In the gut, VHSV significantly increased IFNγ mRNA levels in control fish after 2 and 6 days post-infection, while in the case of fish fed Protec™, this increase was only visible after 6 days of infection (Fig. 5B). After 2 days of infection, VHSV provoked a significant decrease in the transcription levels of this cytokine in the case of fish fed the functional diet (Fig. 5B). Additionally, in fish sampled at day 6, the basal IFNγ mRNA levels were higher in fish fed the supplemented feed than in fish fed the control diet (Fig. 5B).

In gills, the basal IFNγ mRNA levels were higher in fish fed Protec™ than in fish fed the control diet both at day 2 and at day 6 (Fig. 5C). VHSV had no effect on the transcription of this cytokine at day 2 (Fig. 5C), but increased IFNγ mRNA levels both in Protec™- and control-fed fish at day 6 post-infection (Fig. 5C). At this point, the IFNγ transcription levels reached in response to VHSV were higher in fish fed the control diet (Fig. 5C).

3.6. Perforin transcription in kidney, gut and gills of fish fed with Protec™ and challenged with VHSV

To evaluate whether cytotoxic responses were being affected by the viral infection, we also determined the levels of transcription of perforin, a molecule produced by NK cells and cytotoxic T cells that induces pore formation in the cell membranes of target cells [27 6350]. In kidney, VHSV infection significantly increased perforin mRNA levels in animals fed the functional diet after 2 days while it provoked a down-regulation of perforin transcription levels in fish fed the control diet at this point (Fig. 6A). After 6 days of infection, VHSV significantly increased perforin transcription in both control-fed and Protec™-fed fish (Fig. 6A).

In the gut, after 2 days of infection, VHSV induced a significant down-regulation of perforin mRNA levels in fish fed the functional diet, while no effect of the infection was observed in control fish (Fig. 6B). After 6 days of infection, the virus induced a significant up-regulation of

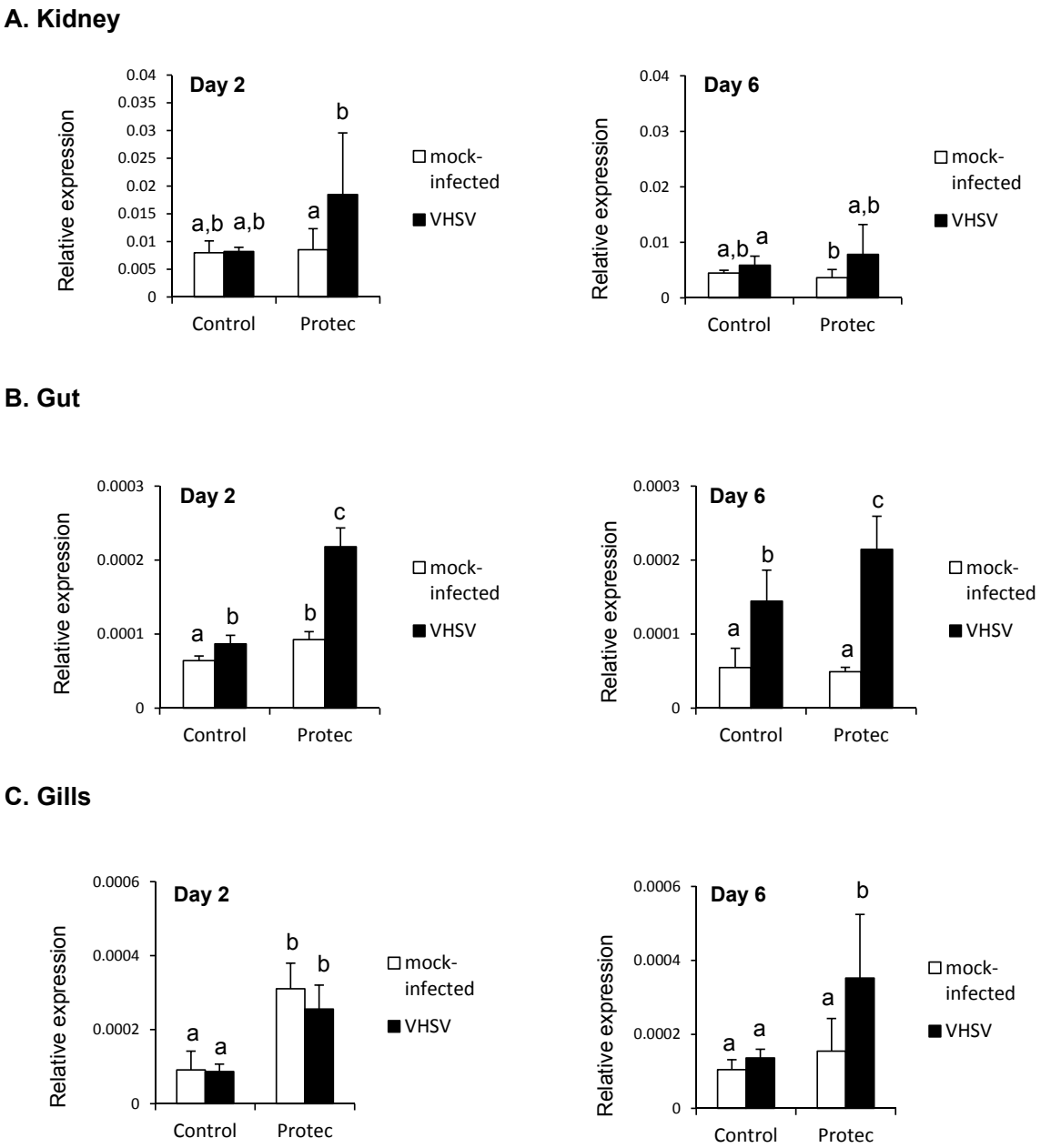

Fig. 3. Transcription levels of IgD in kidney, gut and gill of rainbow trout fed with Protec™ and challenged with VHSV. Rainbow trout were fed and challenged as described in the legend of Fig. 1. At days 2 and 6 post-infection, fish were sacrificed and the levels of transcription of IgD in kidney, gut and gills determined by real-time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF-1α ± SD (n = 4). Different letters denote statistically differences among groups ($P < 0.05$).

perforin transcription levels in control fish but not in fish fed Protec™ (Fig. 6B). However, at this sampling point, basal perforin mRNA levels were higher in fish fed Protec™ than in control fish (Fig. 6B).

In gills, when fish were sampled at day 2, basal perforin mRNA levels were higher in fish fed Protec™ (Fig. 6C). However, at this point, only fish fed the control diet significantly increased perforin mRNA levels in response to VHSV. After 6 days of infection, VHSV infection provoked a significant up-regulation of perforin transcription levels both in fish fed the control or the supplemented (Fig. 6C).

3.7. Total IgM titers in serum

Given that important differences were observed in the levels of IgM transcription in fish fed Protec™ when compared to control fish, we also evaluated the total IgM titers in serum for all experimental groups. We found that total IgM titers were always higher in fish fed Protec™ when compared to fish fed the control diet (Fig. 7). These differences were observed both in control and VHSV-infected groups.

4. Discussion

In the current study, we have demonstrated that the administration of the immunostimulants present in the Protec™ diet can have major effects on the early immune response that is mounted against a viral pathogen. In this experiment, the different diets were fed for 30 days, as previous experiments in our laboratory have established that the results obtained after only 15 days of administration were less marked (data not shown). However, after 30 days of administration, this functional diet had major transcriptional effects in different tissues (kidney, gut and gills) demonstrating that systemic antiviral immune responses and

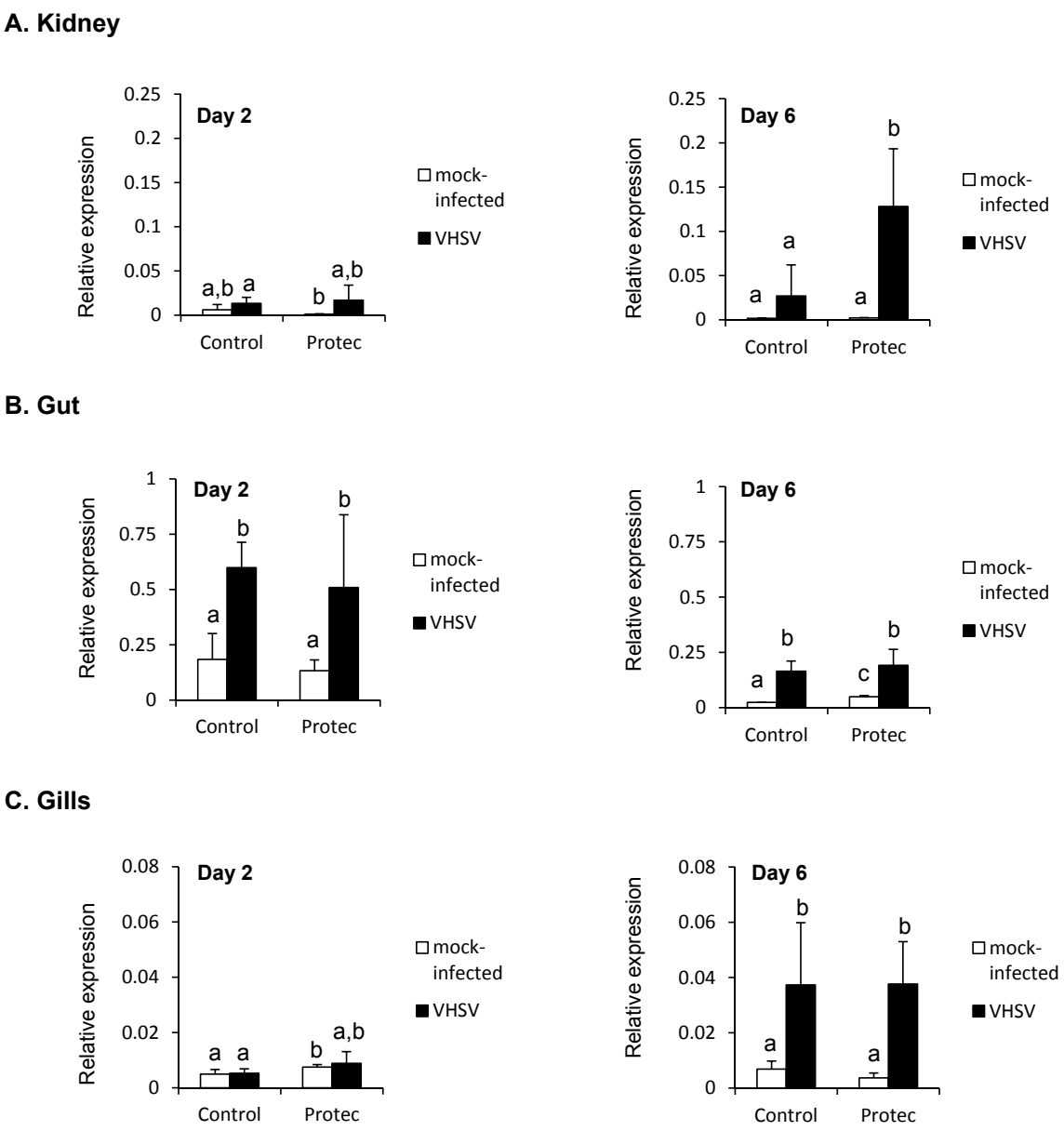

Fig. 4. Transcription levels of Mx in kidney, gut and gill of rainbow trout fed with Protec™ and challenged with VHSV. Rainbow trout were fed and challenged as described in the legend of Fig. 1. At days 2 and 6 post-infection, fish were sacrificed and the levels of transcription of Mx in kidney, gut and gills determined by real-time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF-1α ± SD (n = 4). Different letters denote statistically differences among groups ($P < 0.05$).

those of distant mucosal tissues can be regulated through the feed.

To date, the production of specific IgM antibodies to viral pathogens, including VHSV, has been widely demonstrated in different teleost species [28,29]. However, whether these specific Igs are capable of clearing the virus on their own has not yet been verified. Even though protection against virus has been obtained by passive immunization with serum or IgM purified from infection survivor fish [28,29], specific antiviral IgMs are not detected in serum until 6–10 weeks after infection [28–30], far after the main peak of mortality occurs (generally 1 week after infection). Therefore, specific IgMs produced in response to a viral infection in fish, appear too late to be the unique factor that conditions whether fish naturally survive to the infection. Both in mammals and fish, natural antibodies, non-specific IgMs with low affinity that are constitutively secreted by innate-like B cells, interfere with the early replication of this pathogens to keep the infection under control until a late specific response is mounted [31]. In the current study, we have analysed the transcription of all Ig isotypes present in rainbow trout, namely IgM, IgD and IgT during these early stages, and we have also determined the secretion of IgM in serum through ELISA to study how the diet can affect these natural antibody production. Our results demonstrate that fish fed with Protec™ have an increased concentration of natural IgMs in serum. Furthermore, these animals have an increased capacity to induce IgM, IgD and IgT in kidney, gut and gills in response to the virus than fish fed with the control diet. Presumably, an increase in the production of natural antibodies will correlate with an increased survival to VHSV, given that natural IgMs in trout have been previously shown to interfere with VHSV replication [32]. The effect that the increased induction of IgD found in gut and gills of Protec™-fed animals will have on an overall immune response to the virus is still unpredictable as the precise role that IgD plays is still enigmatic in both fish and mammals [33]. On the other hand, the increased IgT induction observed in these fish fed a supplemented diet should imply greater defence capacities within mucosal surfaces, as IgT has been postulated as an Ig specialized in mucosal defence [34].

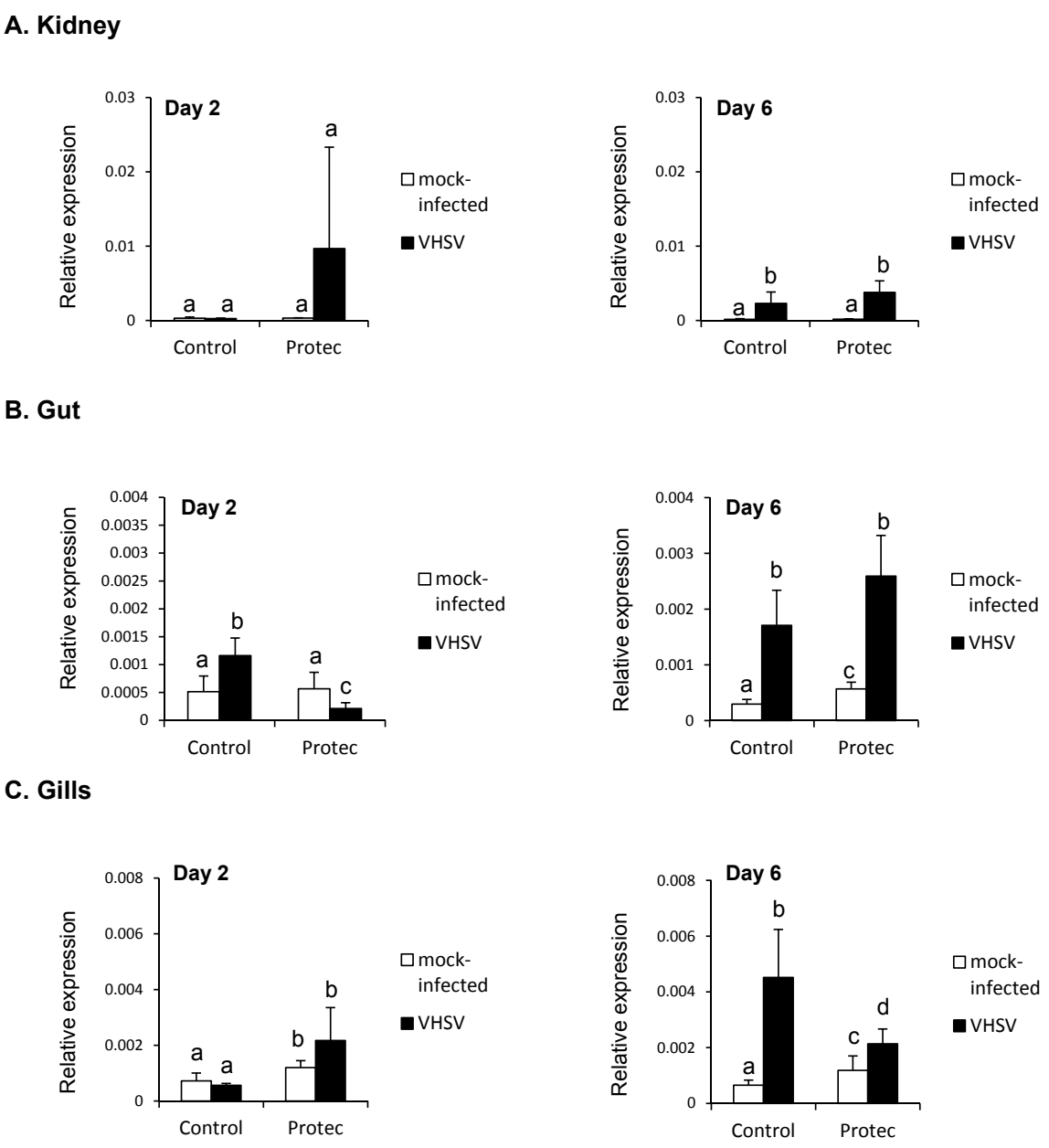

Fig. 5. Transcription levels of IFNγ in kidney, gut and gill of rainbow trout fed with Protec™ and challenged with VHSV. Rainbow trout were fed and challenged as described in the legend of Fig. 1. At days 2 and 6 post-infection, fish were sacrificed and the levels of transcription of IFNγ in kidney, gut and gills determined by real-time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF-1α ± SD (n = 4). Different letters denote statistically differences among groups ($P < 0.05$).

Therefore, the results presented in this work, constitute the first report of increased natural antibody production in response to a supplemented diet in fish.

We also studied the induction of Mx in response to VHSV in fish fed the different diets. Mx proteins are members of a family of genes induced by type I IFN when cells are infected by a virus or stimulated with a virus-like stimuli such as double stranded RNA [35]. These proteins are known to directly interfere with viral replication, effect already demonstrated for Mx in some fish species [36–38], although not yet for rainbow trout. In our work, although Mx was induced by VHSV in kidney, gills and gut, the only difference found between Protec™-fed fish and fish fed with a control diet was observed in the kidney, where fish fed Protec™ reached higher Mx RNA levels in response to VHSV than fish fed the control diet. Similarly, in carp, the administration of β-glucans with the feed provoked an increased Mx transcription when fish were further exposed to poly I:C, at higher levels that those induced by poly I:C in fish fed a control diet [39]. Interestingly, in that study, this effect seemed quite exclusive of Mx since the transcription of other genes studied such as interleukin 1β (IL1β), IL10, tumour necrosis factor α (TNFα) or some chemokines were not influenced by β-glucans [39]. In our study, we also studied the effect of Protec™ in VHSV-induced transcription of IFNγ. In this case, although the virus was capable of transcriptionally up-regulating IFNγ in all tissues and some differential responses were observed in fish fed Protec™, a clear increased induction was not visualized. IFNγ is classified as type II IFN and is generally not responsible for direct antiviral effects such as those exerted by type I IFNs and their induced proteins. IFNγ, on the other hand, plays a role in antiviral defence regulating the action of different cell types, after its secretion by Th1 cells and $CD8^+$ cytotoxic T cells [40]. Additionally, IFNγ is a strong macrophage activator, regulating in these cells MHC I and II surface expression, nitric oxide (NO) production, respiratory burst and their phagocytic capacity [40,41]. Similar results

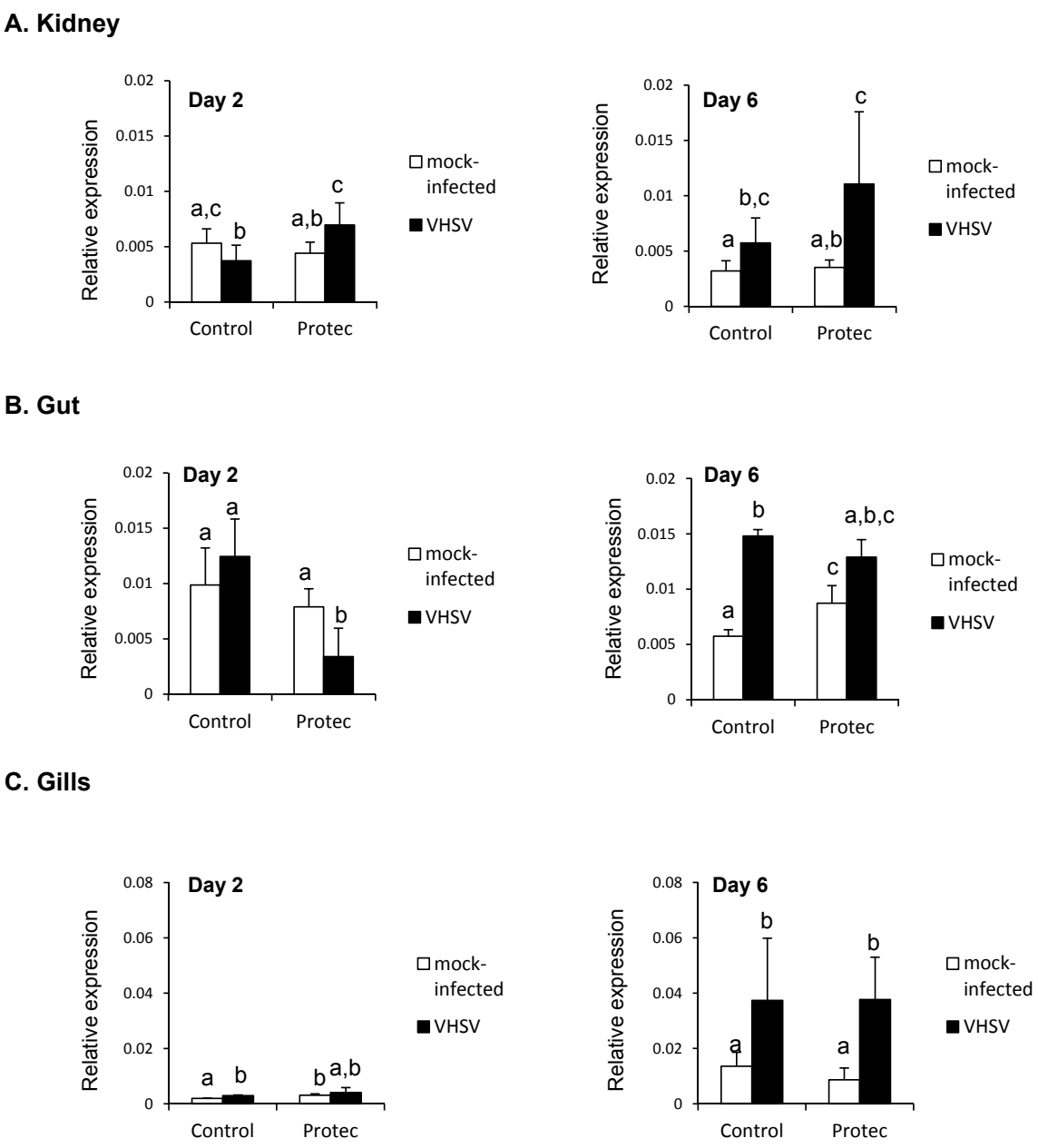

Fig. 6. Transcription levels of perforin in kidney, gut and gill of rainbow trout fed with Protec™ and challenged with VHSV. Rainbow trout were fed and challenged as described in the legend of Fig. 1. At days 2 and 6 post-infection, fish were sacrificed and the levels of transcription of perforin in kidney, gut and gills determined by real-time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF-1α ± SD (n = 4). Different letters denote statistically differences among groups ($P < 0.05$).

were obtained for perforin, as again VHSV induced its transcription in all tissues studied but no significant differences were observed between the inductions observed in fish fed with the different diets.

In conclusion, we have established that the oral administration of a functional diet can have major effects on the antiviral response that is mounted against VHSV not only at a local level (gut), but also in systemic lymphoid organs (kidney) and distant mucosal surfaces (gills). In general, VHSV infection significantly induced the transcription of IgM, IgT, IgD, Mx, IFNγ and perforin in different tissues and at diverse time points. However, when this antiviral response was compared between fish fed a control diet and fish fed Protec™ for 30 days, we found that the main differences were observed in the transcription of all three Ig isotypes. Thus, fish fed Protec™ reached significantly higher Ig transcription levels in response to VHSV than control fish. In correlation with these results, fish fed Protec™ had significantly higher concentrations of non-specific or natural IgMs in serum than control fish. These reveal a previously unreported effect of β-glucans and other immunostimulants on early Ig production in fish, that might help us understand why these functional diets are capable of non-specifically increasing the natural resistance to a viral infection previously reported [18,19]. Finally, the data provided supports the convenience of using functional feeds such as Protec™ as part of health management programs aimed at reducing losses to viral pathogens.

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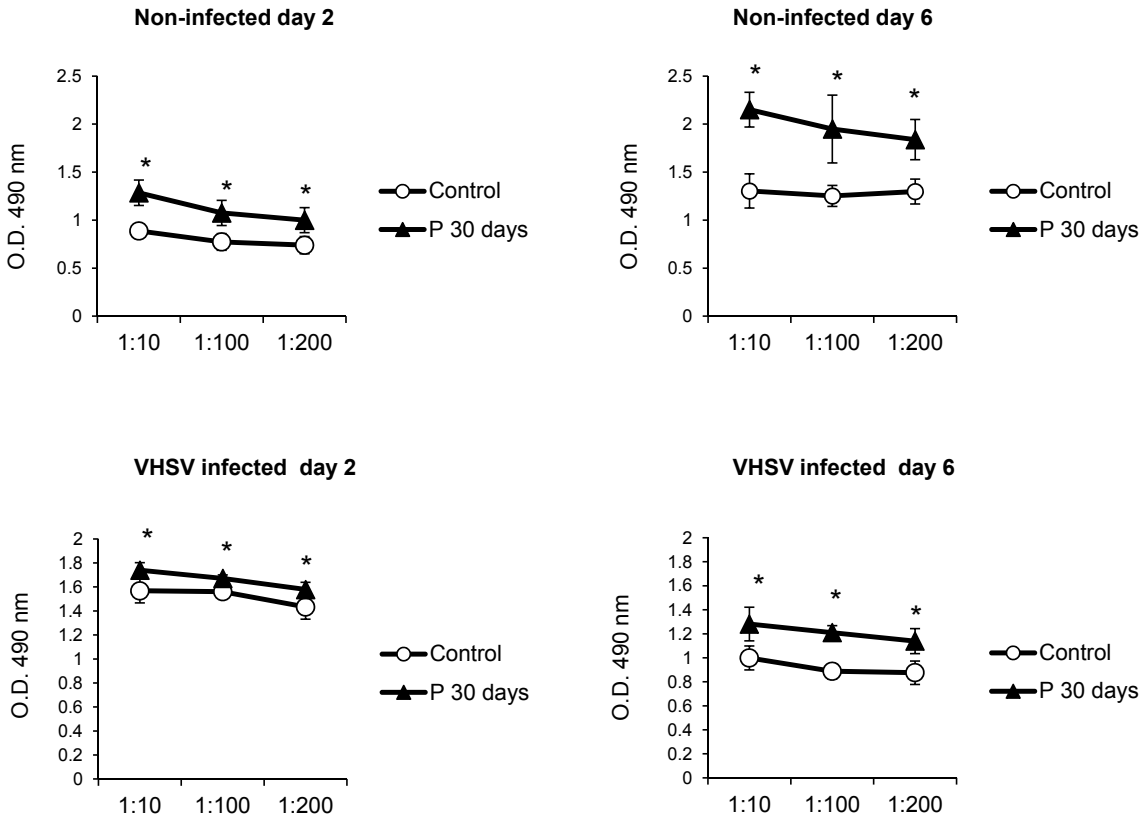

Fig. 7. Total IgM titres in serum. Rainbow trout were fed for 30 days with Protec™ or a control diet. Thereafter, both groups were divided in two and either infected by bath with VHSV (5×10^8 $TCID_{50}$/ml) or mock-infected. At days 2 and 6 post-infection, fish were sacrificed and blood extracted from the caudal vein. Total IgM titres were determined in all groups by ELISA following the protocol detailed in the Materials and Methods section. Data are shown as mean absorbance values at 490 nm ± standard deviation (n = 4). Asterisks indicate IgM titres in fish fed with Protec™ statistically higher than values obtained in fish fed with a control diet ($P < 0.05$).

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