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Inhibition of nervous necrosis virus replication by *Shewanella putrefaciens* Pdp11 extract

Patricia Moreno [a], Daniel Álvarez-Torres [b], M. Carmen Balebona [a], Marta Domínguez-Maqueda [a], M. Ángel Moriñigo [a], Julia Béjar [c], M. Carmen Alonso [a], Esther García-Rosado [a,*]

[a] Universidad de Málaga, Instituto de Biotecnología y Desarrollo Azul, IBYDA, Departamento de Microbiología, Facultad de Ciencias, 29071 Málaga, Spain
[b] Universidad de Málaga, Instituto de Biotecnología y Desarrollo Azul, IBYDA, 29071 Málaga, Spain
[c] Universidad de Málaga, Instituto de Biotecnología y Desarrollo Azul, IBYDA, Departamento de Biología Celular, Genética y Fisiología, Facultad de Ciencias, 29071 Málaga, Spain

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ABSTRACT

One of the most relevant infectious diseases affecting farmed fish is the viral encephalopathy and retinopathy, caused by the nervous necrosis virus (NNV). The two commercial vaccines available against this virus have been designed to protect European seabass against the infection with a particular NNV species, RGNNV. Therefore, the development of strategies to protect different fish species against different viruses is a key issue for the aquaculture industry. In this regard, there are numerous studies focused on the use of probiotics, although their antiviral activity has been poorly investigated. *Shewanella putrefaciens* Pdp11, SpPdp11, is a probiotic with proven positive effects on gilthead seabream and Senegalese sole, protecting those species against several bacterial pathogens; however, its antiviral activity remains to be investigated. The current study is a step forward in the use of probiotics against viral infections, evaluating the anti-RGNNV activity of sonicated-SpPdp11 extracts both, *in vitro* and *in vivo*. According to our results, SpPdp11 extracts compromised RGNNV multiplication in E11 cells, affecting viral assembly and/or exit rather than genome replication. Furthermore, this antiviral activity may be produced by the capacity of the SpPdp11-extract to induce an immune response in treated cells. This anti-RGNNV activity has been confirmed *in vivo*, since 82% of fish fed with the SpPdp11-supplemented diet survived an experimental-RGNNV infection, whereas the survival rate of fish fed with the control diet was 64%. These results suggest that SpPdp11-supplemented feeding can be a promising prophylactic tool against RGNNV and encourage further research on other fish species and viral pathogens.

1. Introduction

Nervous necrosis virus (NNV) is the aetiological agent of one of the most dangerous and widespread fish diseases, viral encephalopathy and retinopathy (VER). This disease is characterized by the vacuolation and necrosis of the central nervous system, causing high mortality rates in early developmental stages (Bandín and Souto, 2020). VER affects a wide number of marine and freshwater fish species worldwide, highlighting its devastating effect on important cultured fish species, such as European seabass (*Dicentrarchux labrax*), gilthead seabream (*Sparus aurata*) and sole (both common sole, *Solea solea*, and Senegalese sole, *S. senegalensis*) (Bandín and Souto, 2020). NNV belongs to *Betanodavirus* genus, *Nodaviridae* family. It is an icosahedral, non-enveloped virus, with two single-stranded positive-sense RNA segments, RNA1 and RNA2. Based on the variable T4 region within RNA2, NNV has been classified into four genotypes, considered as species by the International Committee on Taxonomy of Viruses (ICTV) (Sahul Hameed et al., 2019): striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). RGNNV and SJNNV are the viral species more often detected in Southern Europe (Bandín and Souto, 2020).

Due to the economic impact of viral diseases on aquaculture, the development of preventive measures and/or treatments is a main priority for the industry. Although there are an increasing number of studies on vaccines against NNV infections, only two formalin-

* Corresponding author at: Department of Microbiology, Faculty of Sciences, University of Malaga, Campus Teatinos, 29071 Malaga, Spain.
E-mail addresses: patriciamgarcia@uma.es (P. Moreno), dani-alto@uma.es (D. Álvarez-Torres), balebona@uma.es (M.C. Balebona), martadm@uma.es (M. Domínguez-Maqueda), morinigo@uma.es (M.Á. Moriñigo), bejar@uma.es (J. Béjar), mdalonso@uma.es (M.C. Alonso), megarciar@uma.es (E. García-Rosado).

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inactivated vaccines for seabass vaccination against RGNNV have been commercialised (ALPHA JECT micro® 1Noda, Paharmaq and ICTHIO-VAC® VNN, Hipra) (review in Valero et al., 2021). Furthermore, there are no studies about NNV vaccines able to protect different fish species against different betanodavirus; in addition, fish are especially susceptible to VER at early stages of development, when they still are not immunocompetent and they cannot be vaccinated by injection.

In this scenario, antiviral compounds represent an alternative to prevent and/or to treat fish viral diseases (Pereiro et al., 2021). Specifically, the development of functional feeds (with additives that improve fish health) has become one of the main targets for the aquaculture industry. In this regard, there are numerous studies focused on the use of probiotics, since they are immunostimulants, and provide widely proven protection against bacterial infections; however, their antiviral activity has only been reported in a few studies (Simón et al., 2021).

Shewanella putrefaciens Pdp11, known as SpPdp11, is a probiotic isolated from the skin of heathy gilthead seabream (Chabrillón et al., 2005). It has positive effects on gilthead seabream and Senegalese sole metabolism, nutrition, growth, immune response, and stress response (Cámara-Ruiz et al., 2020), protecting against several bacterial pathogens, such as *Vibrio harvey, Photobacterium damselae* subsp. *piscidida,* and *Listonella anguillarum* (Chabrillón et al., 2005, 2006). However, the antiviral activity of this probiotic is still unknown.

Probiotics are live microorganisms; therefore, their use in diets has raised safety concerns, and their industrial processing and storage can be challenging. For these reasons, the interest in non-viable probiotics that maintain their properties has increased in last years (Nataraj et al., 2020). In this sense, two probiotic-related concepts are being used, paraprobiotic and postbiotic. Paraprobiotic comprises inactivated cells (intact or ruptured) and crude cell extracts (Siciliano et al., 2021), whereas postbiotic refers to metabolic products secreted by probiotics (Peluzio et al., 2021). However, since there is no definition recommended by international regulatory administrations, the term postbiotic is widely used for both concepts, postbiotics and paraprobiotics (Nataraj et al., 2020).

In the present study, the effect of a postbiotic, SpPdp11-sonicated extract, on the *in vitro* replication of an RGNNV isolate highly virulent to European seabass has been evaluated. Besides, its ability to stimulate the immune antiviral response of E11 cells was tested. Finally, the effect of the dietary administration of SpPdp11-sonicated extract on the resistance of juvenile seabass to RGNNV has also been examined.

2. Material and methods

2.1. Cell culture and virus

E11 cells (Iwamoto et al., 2000) were grown in 25-cm^2 flasks (Nunc, ThermoFisher) at 25 °C using Leibovitz L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 10 mg/ml streptomycin (Sigma) (growth medium).

The viral isolate SpDl_IAusc965.09 (RGNNV, Moreno et al., 2019) was propagated on E11 cells at 25 °C with L-15 medium supplemented with 2% FBS, 100 units/ml penicillin and 10 mg/ml streptomycin (maintenance medium) until fully cytopathic effect (CPE) appearance. The viral suspension obtained was titrated following the 50% tissue culture infective dose method ($TCID_{50}$) (Reed and Müench, 1938) and stored at −80 °C until used.

2.2. Obtention of Shewanella putrefaciens Pdp11 extract

The probiotic strain SpPdp11 was grown in trypticase soy broth (TSB, Sigma) supplemented with 1.5% NaCl (TSBs) according to Tapia-Paniagua et al. (2010). The culture was incubated at 23 °C in an orbital shaking until exponential phase (1 optical density, OD, value at 600 nm). At that moment, cells were collected by centrifugation at 3000 ×*g* for 10 min at 4 °C, and adjusted to 1.7 OD (2×10^9 cfu/ml) in phosphate buffered saline (PBS, Sigma).

This bacterial suspension was sonicated using the UP200S ultrasonic processor (Hielscher). The sonication conditions were 4 pulses of 30 s with an amplitude of 35 and cycle setting of 1. The resulting SpPdp11 extract was cleared by centrifugation, at 3000 ×*g* for 5 min at 4 °C, total amount of proteins was 0.4 μg/μl, quantified by Qubit fluorometric analyses (ThermoFisher), and checked for the absence of viable cells by seeding on TSAs plates (24 h at 23 °C) and on E11 cells in maintenance medium (72 h at 23 °C). The final SpPdp11 extract was stored at −20 °C until used.

2.3. Evaluation of SpPdp11-extract effects on E11 cells

2.3.1. Cytotoxicity assay

Monolayers of E11 cells grown on 96-well plates were incubated in maintenance medium containing different concentrations of SpPdp11 extract (total protein concentrations ranging from 2×10^{-1} to 1.24×10^{-5} μg/μl). Non-treated cells were used as control. After 72-h incubation at 25 °C, cell survival was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma)-reduction assay (Moreno et al., 2018). Optical density was quantified at 550 nm using the Whittaker Microplate Reader 2001. The concentration with an OD value not significantly lower than that of the control group was considered as the maximal safety postbiotic concentration (no-cytotoxic) (Nguyen et al., 2012).

2.3.2. Cellular growth kinetics analysis

E11 cells (2.5×10^3 cells/ml) were cultured on 24-well plates in growth medium at 25 °C. After 24-h incubation, the medium was removed and immediately replaced by growth medium containing 8×10^{-3} μg/μl SpPdp11 extract. These cells were incubated for 6 h. Following this incubation period, SpPdp11-containing medium was removed, cells were washed with PBS, and SpPdp11-free growth medium was added. An additional experimental group, consisting of cells exposed to SpPdp11 extract during all the experimental time (7 days) was also set up. In all the experimental groups, cells from three wells were collected at 1, 3, 5 and 7 days to be counted, in triplicate, using a Z1 coulter (Beckman Coulter). Non-treated cells were used as control.

2.3.3. Immunomodulation assay

The ability of SpPdp11 extract to induce an immune response in E11 cells was evaluated by the transcription quantification of the genes mx dynamin like GTPase (*mx*), toll-like receptor 3 (*tlr3*), ubiquitin ligase E3 (*e3*), tumour necrosis factor alpha (*tnfα*) and heat shock protein 70 (*hsp70*). Monolayers of E11 cells seeded on 24-well plates were cultured in maintenance medium with 8×10^{-3} μg/μl SpPdp11 extract. Cells from three wells were collected at 6, 24, 48 and 72 h after the start of the treatment. Untreated cells were used as control. Total RNA was extracted with the E.Z.N.A. total RNA Kit I (Omega Bio-Teck) following manufacturer guidelines. RNA was treated with DNase I Recombinant from bovine pancreas (Roche). Briefly, 1× Incubation Buffer and DNase I Recombinant (10 U) were mixed in a 25-μl reaction. This mixture was incubated at 37 °C for 30 min, followed by incubation at 75 °C for 10 min to stop the reaction. After this treatment, RNA (1 μg) was reverse-transcribed with the Transcriptor First Strand cDNA synthesis kit (Roche) in a reaction carried out in two steps. In a first step, RNA and 60 μM random hexamer primers were mixed in a 13-μl reaction, followed by incubation at 65 °C for 10 min. In a second step, the remaining components were added up to 20 μl. These components were: 1× Transcriptor Reverse Transcriptase Reaction Buffer, Protector RNase Inhibitor (20 U), 1 mM Deoxynucleotide Mix, and Transcriptor Reverse Transcriptase (10 U). This mixture was incubated at 25 °C for 10 min, followed by 50 °C for 60 min and 85 °C for 5 min. Relative quantitative PCRs (qPCRs) were conducted in a LightCycler 96 Thermocycler following the conditions described by Moreno et al. (2022) and using the

primers specified in Table 1. All the amplifications were conducted in 20-μl mixtures containing cDNA generated from 50 ng of RNA, 1× Fast Start Essential DNA Green Master Mix (Roche) and 10 pmol specific primers (Table 1). Amplification conditions were 95 °C for 10 min, and 45 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Melting curves were obtained at 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s. Relative fold change values were calculated according to Plaffl (2004), using *beta-actin* as endogenous reference gene (Table 1).

2.4. In vitro antiviral activity of SpPdp11 extract

The antiviral activity was evaluated in E11 cells following three different procedures: (i) neutralization assay, (ii) 6-h pre-adsorption assay, and (iii) post-adsorption assay. The protein concentration of SpPdp11 extract used in all the experiments was 8×10^{-3} μg/μl, and RGNNV was always inoculated at 10^3 $TCID_{50}$/ml. All analyses were conducted in triplicate.

For the neutralization assay, SpPdp11 extract and RGNNV (both in inoculation medium) were mixed (1:1, v:v). The resulting mixture was incubated for 1 h at 25 °C. Afterwards, this mixture was used to inoculate E11 monolayers. After 1-h incubation (viral adsorption period), the mixture was removed, cells were washed with PBS, and maintenance medium was added.

Regarding the 6-h pre-adsorption treatment: E11 cells were treated with SpPdp11 extract (6 h at 25 °C), washed with PBS, and subsequently inoculated with the virus. After viral adsorption, viruses were removed, and maintenance medium was added.

The procedure for the post-adsorption assay was as follows: E11 cells were infected as described previously. After viral adsorption, viral suspension was removed and replaced by maintenance medium containing SpPdp11 extract.

Two control groups were included in each experiment: infected cells non-treated with SpPdp11 extract (viral control group), and non-treated non-infected cells (cell control group).

In each set of experiments, cell survival was determined at 7 days post-inoculation (p.i.) by MTT addition, as described in 2.3.1., and virus inhibitory rate was calculated according to the following formula:

$$[(OD_{treatment+virus} - OD_{no-treatment+virus})/(OD_{no-treatment-virus} - OD_{no-treatment+virus})] \times 100$$

In addition, viral replication (viral genome and infective viral particle production) was also analysed. At 0, 24, 48 and 72 h p.i., cells and supernatants were separately collected and used for viral genome quantification and viral titration, respectively.

RNA extraction and cDNA synthesis have been performed as described above. Protocol for viral genome quantification by absolute qPCR was that described by Moreno et al. (2019). Primers used for absolute qPCR were RG_RNA2 F4/RG_RNA2 R1 (Table 1). Serial dilutions of the pJET vector (Thermo) containing the RGNNV RNA2 sequence were used for the reference standard curve (Moreno et al., 2016). Virus titration was calculated by $TCID_{50}$.

2.5. In vivo antiviral activity

2.5.1. Feeding trial

Two hundred juvenile seabass (6.14 g, average weight) were acclimated for 2 weeks in a re-circulating system in the Marine Fish Facilities of CTAQUA, Cádiz (Spain). Temperature and salinity were 22 ± 1 °C and 38 g/l, respectively. The photoperiod was 12 h light: 12 h dark, and animals were fed during this period with a commercial pellet diet (Skretting, Spain). The absence of betanodavirus carriers was tested as described by Lopez-Jimena et al. (2011, 2012).

After acclimatization, two fish groups with different feeding regimes were stablished: a control group, receiving commercial feed, and an experimental group, which was fed with commercial pellet supplemented with SpPdp11 extract (6.3 μg total amount of proteins/g pellet, LifeBioencapsulation). Daily ration of feed in both groups was 2% biomass. Animals were fed for 30 days. At that time, fish weight was recorded to calculate growth and feed efficiency parameters: weight gain (WG = final mean weight g − initial weight g), feed intake (FI = Σ[(total feed consumption (g))/(number of fish)]/number of days), feed conversion ratio (FCR = feed intake g/weight gain g), and specific growth rate (SGR = [(Ln final weight − Ln initial weight)/number of days] × 100). Fish were handled according to the European Union guidelines for the handling of laboratory animals (Directive 2010/63/UE) and the Spanish directive (RD53/2013). To minimize fish suffering, trials were accomplished in accordance to the Bioethics Committee of the Junta de Andalucia (number: 15/06/2020/076).

2.5.2. RGNNV challenge

After the feeding period, fish were distributed in four experimental groups ($n = 25$ each): (i and ii) specimens fed with or without SpPdp11-extract diet and inoculated with RGNNV, (iii and iv) fish fed with or without SpPdp11-extract supplemented diet and inoculated with L-15, as negative controls. Infections were performed by intramuscular injection with 3.8×10^5 $TCID_{50}$/g fish. Fish were daily monitored to record clinical signs and mortality for four weeks. The challenge was performed in duplicate.

2.6. Statistical analyses

All data, except those related to mortality, were analysed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Normality distribution was verified by the Shapiro-Wilk test. The one-way ANOVA was the statistical test used, with the Tukey's multiple comparison analysis as post-hoc test. Values of $p < 0.05$ were considered significant.

Table 1
Primers used in this study.

Gene	Sequence (5′-3′)	Amplicon size (bp)	Reference
Beta-actin	F: CACTGTGCCCATCTACGAG	200	Chen et al. (2017)
	R: CCATCTCCTGCTCGAAGTC		
mx	F: GGGGTCAGAAGGAGATCACA	150	Poisa-Beiro et al. (2008)
	R: ATGATGCACCAGCTCAAGTG		
tlr3	F: TGCAACACTCCACTGACTTACTTTAA	115	Moreno et al. (2018)
	R: AGGACAGCTGTGCTAAGTATATAA		
e3	F: TGCACTTGCAAGGCTGTCA	100	Moreno et al. (2018)
	R: CTCCTAGGATACTTGCATAGAAGACAAC		
hsp70	F: GTCGTGGATCTGTCCCTTGT	98	Purohit et al. (2014)
	R: CTCGCTTTGAGGAGCTGTG		
tnf-α	F: TCCAAGGCAGCCATCCATTT	108	This study
	R: TGTTGTTCACCAGCCTGAA		
RGNNV-RNA2	F: ACCGTCCGCTGTCTATTGACTA	126	Moreno et al. (2016)
	R: CAGATGCCCCAGCGAAACC		

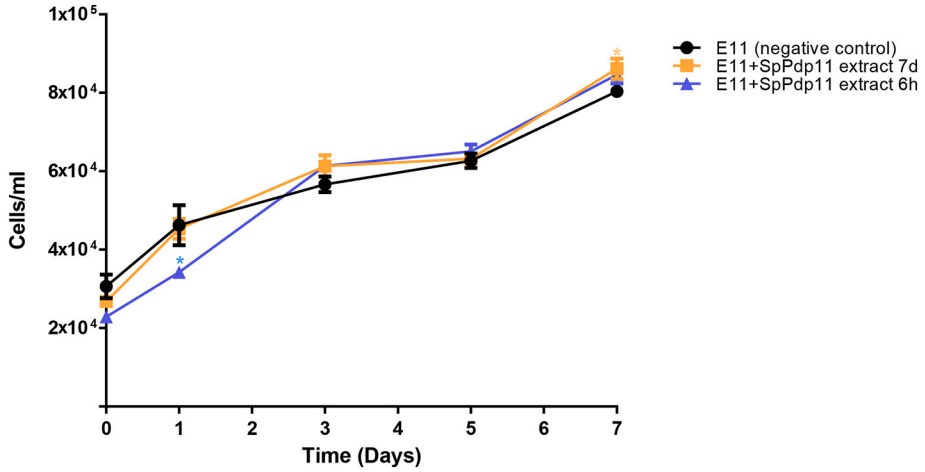

Fig. 1. Effect of SpPdp11 extract on E11 cells. Growth curves of E11 cells treated with SpPdp11 extract for different time periods, 6 h and 7 days. Non-treated cells were used as control. Asterisks indicate significant differences between each experimental condition and control cells at the same time point ($p < 0.05$) Results are mean ± standard deviation (SD) ($n = 3$).

3. Results

3.1. Effect of SpPdp11 extract on E11 cells

The maximal non-cytotoxic concentration of the extract was 2.5 × 10^{-2} µg/µl. However, to ensure lack of cytotoxicity, a lower concentration (8 × 10^{-3} µg/µl) was used for further assays.

The effect of the postbiotic on cellular growth was determined by comparing the growth profiles of untreated and treated cells. E11 cells were treated with the postbiotic during different time periods (Fig. 1). The maintenance of the postbiotic on the cells throughout the assay period (7 days) did not provoke significant differences in cell concentrations between treated and non-treated groups from 1 to 6 days. In contrast, at 7 days a significantly higher concentration of cells was observed in the postbiotic-exposed group (8.6 × 10^4 cell/ml). The treatment of E11 cells for 6 h did not change the growth curve; the only significant difference between control and exposed cells was recorded at 1 day, when a significantly lower concentration of cells was recorded in treated cells (3.4 × 10^4 cell/ml) compared to the control group (4.6 × 10^4 cell/ml).

3.2. SpPdp11 extract modulates the transcription of E11 immune-related genes

The transcription of *mx*, *hsp70*, *tnfα*, *e3* and *tlr3* was quantified at 6, 24, 48 and 72 h. As it is represented in Fig. 2, the highest significant induction was obtained for *mx* gene (110.34 FC at 24 h, $p < 0.01$). The postbiotic induces an early transcription of *hsp70* and *mx* (at 6 and 24 h, respectively), whereas a delay in the induction of the remaining genes was recorded. Thus, *tnfα* was significantly induced only at 48 h (6.18 FC $p < 0.05$), whereas *e3* and *tlr3* induction was maintained from 24 h onwards (Fig. 2).

3.3. In vitro anti-RGNNV activity of SpPdp11 extract

To evaluate the antiviral activity of the SpPdp11 extract, the virus inhibitory rate in E11 cells was evaluated after three different treatments: viral neutralization, 6-h pre-adsorption treatment, and post-adsorption treatment. According to the results shown in Fig. 3, the postbiotic does not neutralize the virus. In contrast, the treatment of E11 cells for 6 h before viral infection caused a strong inhibition of virus effect (67.3%) (Fig. 3). Moreover, the post-adsorption treatment of infected cells also provoked an important viral inhibition (55%). Thus,

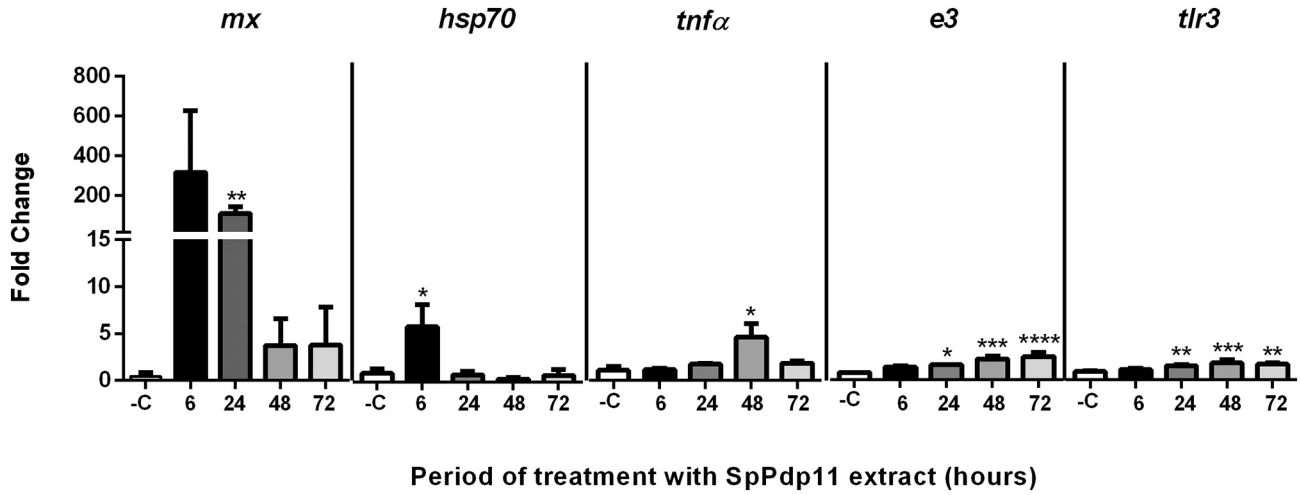

Fig. 2. Immunomodulatory effect of SpPdp11 extract on E11 cells. Relative quantification of *mx, hsp70, tnfα, e3* and *tlr3* transcription respect to beta-actin in postbiotic-treated E11 cells. Asterisks indicate significant differences compared to the control group (* $p < 0.05$; ** < 0.01; *** < 0.001; **** < 0.0001). Results are mean ± standard deviation (SD) (n = 3).

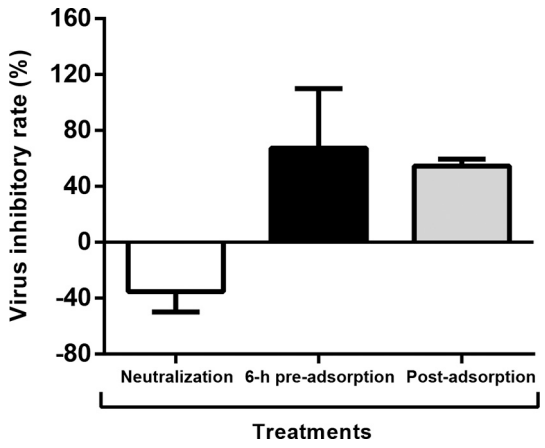

Fig. 3. Anti-RGNNV activity of SpPdp11 extract on E11 cells represented as virus inhibitory rate: $[(OD_{treatment+virus}\text{-}OD_{no\text{-}treatment+virus})/(OD_{no\text{-}treatment\text{-}virus}\text{-}OD_{no\text{-}treatment+virus})] \times 100$. Viral inhibition was determined on RGNNV-infected E11 cells treated with the postbiotic at different conditions: viral neutralization, 6-h pre-adsorption treatment, and post-adsorption treatment. Results are mean ± standard deviation (SD) (n = 3).

SpPdp11 extract protects the cells from RGNNV infection.

To further study the antiviral activity of SpPdp11 extract, inhibition of both, viral genome replication, and infective viral particle production, was determined after 6-h pre- adsorption and post-adsorption treatments. Results are shown in Fig. 4.

The 6-h pre-adsorption treatment provoked a non-significant decrease of viral genome replication. In contrast, the post-adsorption treatment provoked a significant decrease in viral genome replication, from 24 h p.i until 72 h p.i., when viral genome decreases in a 11.9% compared to control (Fig. 4A). Interestingly, the inhibition of viral titre (determined only at 48 h p.i.) was high for both treatments, 33.2% and 46% for pre-treatment and post-treatment assays, respectively (Fig. 4B). Although the difference with the non-treated control was only significant in the post-treatment assay.

3.4. *In vivo antiviral effect of SpPdp11-extract dietary administration*

No negative effect on juvenile sea bass growth was observed after feeding for 30 days with the postbiotic-supplemented diet, since non-significant differences were recorded ($p < 0.05$) for any of the growth parameters analysed (weight gain, specific growth rate, feed intake and feed conversion ratio) (Table 2).

In order to evaluate the antiviral effect of the postbiotic dietary

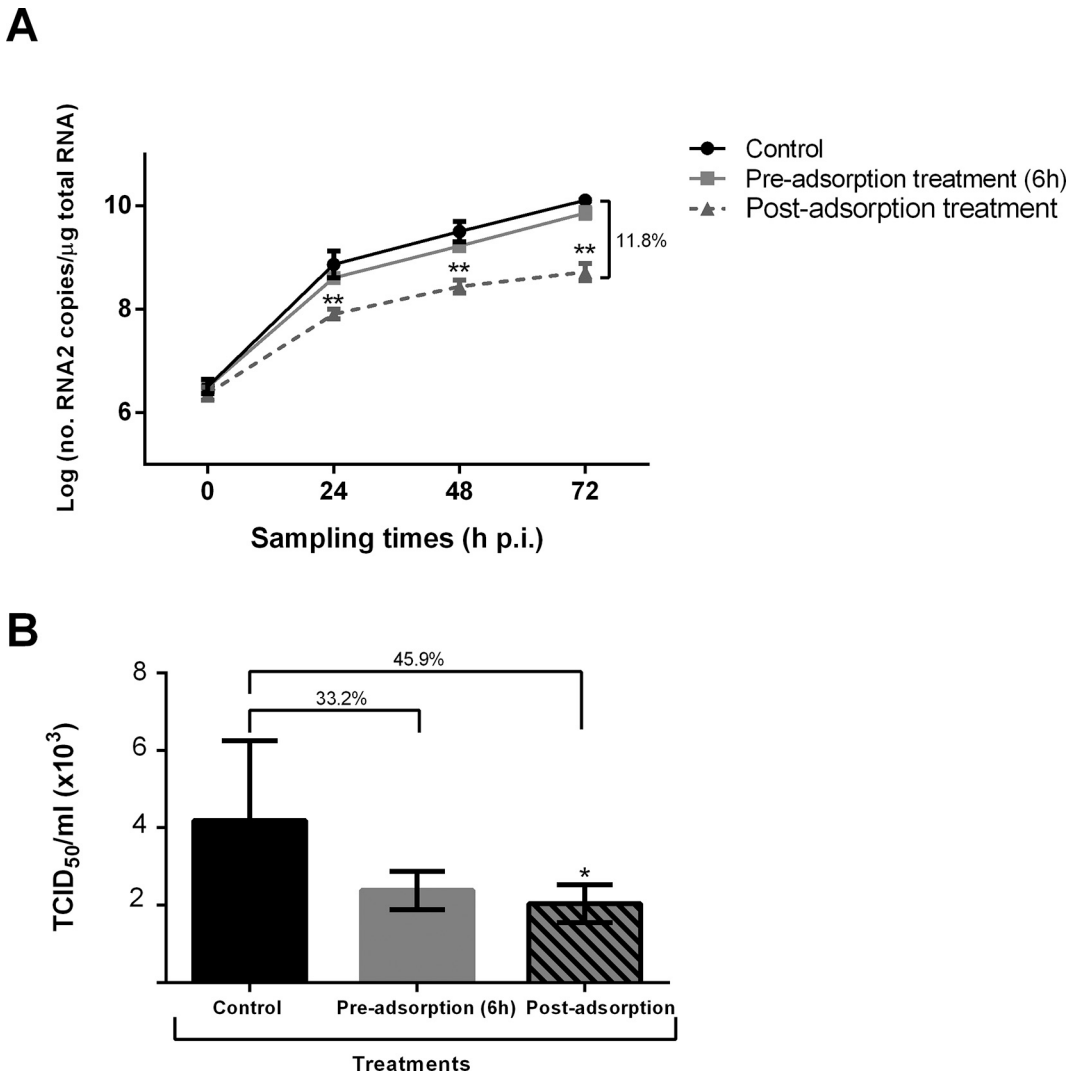

Fig. 4. Effect of SpPdp11 extract on RGNNV multiplication in E11 cells. (A) Absolute quantification of viral RNA2. (B) Quantification of extracellular infective viral particles ($TCID_{50}$/ml) at 48 h p.i.. Asterisks indicate significant differences compared to the control group (* $p < 0.1$; ** < 0.05). Results are mean ± standard deviation (SD) (n = 3).

Table 2
Growth parameters of juvenile sea bass fed during 30 days with a commercial diet (control diet) (n = 100) or postbiotic-supplemented diet (n = 100). No significant differences were detected between both groups.

Parameters	Treatments	
	Control diet	Postbiotic-supplemented diet
Initial weight (g)	6.13 ± 0.84	6.14 ± 0.85
Final weight (g)	10.67 ± 1.52	10.13 ± 1.3
Weight gain (g)	4.54 ± 0.02	3.99 ± 0.03
Weight gain (%)	74.01 ± 0.16	64.89 ± 0.55
Feed intake (FI) (g/fish/day)	0.160 ± 0.001	0.156 ± 0.001
Feed conversion ratio (FCR)	0.99 ± 0.01	1.10 ± 0.02
Specific growth rate (SGR) (%/day)	1.98 ± 0.005	1.79 ± 0.01

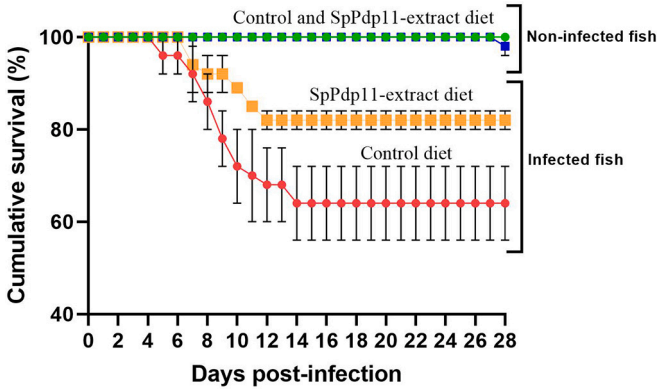

Fig. 5. Cumulative survival of RGNNV-infected seabass fed with a SpPdp11-extract supplemented diet. Results are mean ± standard deviation (SD) (n = 2).

administration, fish fed for 30 days were challenged with an RGNNV isolate highly pathogenic to seabass. Results were expressed as cumulative survival (Fig. 5). Survival rates were analysed using the Kaplan-Meyer test (Supplementary Fig. 1), and the statistical comparison between survival distributions was carried out using a log-rank Mantel Cox analysis. At four weeks p.i., 82% of fish fed with the postbiotic-supplemented diet survived to RGNNV infection, whereas the cumulative survival in the group of fish fed with the control diet was significantly lower (64%, $p < 0.0001$) (Supplementary Fig. 1). Clinical symptoms were similar for both groups (anorexia, loss of appetite and abnormal swimming). Mortality began earlier (at 5 days p.i.) in the commercial diet group, dying in an exponential way until 14 days p.i.; whereas the onset of mortality in the postbiotic group was delayed two days, and mortality ended earlier, at 12 days p.i.. Thus, these fish showed an increased resistance to RGNNV infection. No mortality was observed in any of the un-infected control groups considered.

4. Discussion

In the present study, the antiviral activity of crude extracts from the fish probiotic SpPdp11 has been determined against the infection with the betanodavirus RGNNV. For the *in vitro* studies, a previous analysis on the effect of SpPdp11 extracts on E11 growth has been conducted. To this end, cells were treated with the postbiotic for different time periods: 6 h and 7 days. The postbiotic did not affect cell growth, although it should be highlighted that the final concentration of cells was maximal ($p < 0.05$) when the postbiotic was maintained on the cells throughout the assay (7 days) (Fig. 1). This result may indicate that the probiotic extract induces cell proliferation *in vitro*. In fact, this result is supported by outcomes published by Chen et al. (2020), who described that dietary administration of SpPdp11 facilitated wound closure in gilthead seabream experimentally injured by re-epithelization of the damaged skin.

On the other hand, postbiotic dietary administration did not have negative effects on seabass growth (Table 2), although an increase in growth parameters were neither detected. According to Waiyamitra et al. (2020), feeding periods of *ca.* 60 days are required to observe an increase in animal growth performance. Furthermore, it seems that live probiotic diets have higher capacity to improve fish growth than non-live supplemented diets (Domínguez-Maqueda et al., 2021).

The treatment of E11 cells with extracts of SpPdp11 induced the transcription of *hsp70* as early as 6 h; however, this transcription returned to basal levels from 24 h onwards. The heat shock 70-kDa protein (HSP70) belongs to the heat shock protein (HSP) family, and it has been described as an indicator of cellular stress in several groups of animals, including fish (Cara et al., 2005). Therefore, the *hsp70* transcription profile suggests an early SpPdp11-derived cellular stress; however, this effect is rapidly overcome, indicating that long-lasting treatment with SpPdp11 extracts does not produce cellular stress. Similar results have been previously obtained *in vivo*; thus, Domínguez-Maqueda et al. (2021) described that *hsp70* and *hsp90aa* genes were not transcribed in intestine of Senegalese sole fed with diets supplemented with SpPdp11, both alive or inactivated.

Furthermore, in the present study, the pro-inflammatory gene *tnfα* was transcribed only at 48 h. The transcription of this gene has also been described in several previous reports, involving head-kidney leukocytes of European seabass treated with extracellular products of the probiotic *Vagococcus fluvialis* L21 (Román et al., 2015), or *in vivo* experiments with different probiotic cells, both alive and dead (Caipang et al., 2008; Domínguez-Maqueda et al., 2021; Giri et al., 2015, 2016; Panigrahi et al., 2007; Yan et al., 2016). In particular, pro-inflammatory cytokines may activate the immune response. Inflammation is essential to control NNV infection; however, an uncontrolled inflammatory process could provoke drastic negative effects on fish health (Gémez-Mata et al., 2021). Therefore, the short and low transcription of *tnfα* observed in the present study could indicate a role in the activation of the immune response that might be involved in the control of subsequent infections, including betanodavirus infections.

The transcription of genes involved in the antiviral response (*mx*, *tlr3* and *e3*) was also increased after treating the cells with SpPdp11 extract. TLR3 is an RNA viral receptor, E3 is involved in ISGylation and ubiquitination, and Mx is a key element within the type I interferon (IFN I) system. The induction of IFN I-related genes by probiotics or probiotic-derived substances has been widely described both, *in vitro* and *in vivo* (Jaramillo-Torres et al., 2019; Román et al., 2015; Waiyamitra et al., 2020; Wu et al., 2020). Specifically, Wu et al. (2020) described *mx* induction after treatment of cBB cells (derived from barramundi, *Lates calcarifer*) with the supernatant of a *Shewanella* strain culture. Furthermore, the expression of *mx* in this cell line caused a decreased NNV replication. Therefore, the results obtained in the present study may indicate that the extract of SpPdp11 may establish an antiviral state that would protect cells from viral infections. This hypothesis has been corroborated by several antiviral assays using different approaches. Thus, SpPdP11 treatment caused virus inhibition (*ca.* 50%, Fig. 3), but only during the pre-adsorption and post-adsorption periods. These results are similar to those described by Wu et al. (2020), who reported that supernatant of *Shewanella* culture should be in contact with the cells during the course of the assay in order to exert anti-NNV activity.

The *in vitro* analysis of viral replication revealed that SpPdp11 extract seems to affect viral assembly and/or exit rather than viral genome replication. Thus, a significant inhibition of viral titre of 46% was observed when the postbiotic was added after viral adsorption (Fig. 4B), whereas, the inhibition of viral genome replication was lower (11.8%) for the same experimental approach. The interference in viral assembly could be caused by proteins encoded by IFN-stimulated genes (ISGs), such as Mx, which exerts antiviral activity by interaction with viral proteins, accumulating them in cellular compartments (Kochs

et al., 2002). However, further experiments are necessary to elucidate the underlying mechanisms involved in the antiviral activity exerted by SpPdp11 extract.

Regarding the *in vivo* antiviral activity, the present study describes the anti-RGNNV activity of SpPdp11 extract in seabass. The positive effects of SpPdp11 on gilthead seabream and Senegalese sole are widely reported (review in Cámara-Ruiz et al., 2020); however, this is the first study describing the effect of an extract derived from this fish probiotic on seabass, and, more importantly, describing its capacity to establish an antiviral state able to protect this fish species from betanodavirus infections. Antiviral activity exerted by probiotics has been poorly described in fish to date; however, they have a great potential in aquaculture, since there are no treatments against most of fish viral infections, they induce an unspecific innate immune response, which can be effective against different viral pathogens, and they are easy to use as feed supplements. All these characteristics make probiotics in general, and SpPdp11 in particular, one the most promising tools in the defence against viral infections in fish farming.

In conclusion, this study has demonstrated that sonicated extracts of the probiotic SpPdp11 exert antiviral activity, both *in vitro* and *in vivo,* against a betanodavirus highly virulent to seabass, an RGNNV isolate. According to our results, to exert anti-RGNNV activity *in vitro,* the postbiotic needs to be in contact with the cells, inducing an immune response that could be involved in the antiviral activity, affecting mainly viral assembly and/or exit. These results pave the way for the use of SpPdp11 postbiotic as functional feed in seabass farming as a strategy to increase its resistance to RGNNV, and also encourage further research aimed at testing its effect on other fish species and against other viral pathogens.

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CRediT authorship contribution statement

Patricia Moreno: Investigation, Methodology, Formal analysis. **Daniel Álvarez-Torres:** Investigation, Methodology, Formal analysis. **M. Carmen Balebona:** Conceptualization. **Marta Domínguez-Maqueda:** Methodology. **M. Ángel Moriñigo:** Conceptualization, Writing – review & editing. **Julia Béjar:** Conceptualization, Writing – review & editing, Funding acquisition. **M. Carmen Alonso:** Conceptualization, Writing – review & editing. **Esther García-Rosado:** Conceptualization, Writing – original draft, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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