Tetraselmis suecica and Porphyridium cruentum exopolysaccharides show anti-VHSV activity on RTG-2 cells

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Abstract

The use of functional feed additives is an important approach to both, prevent and fight, viral diseases in aquaculture. In this regard, microalgae-derived products, and, more specifically, microalgal exopolysaccharides (EPSs), have attracted attention, since multiple biotechnological applications are being described for these molecules. Furthermore, depending on culture conditions, the composition and, therefore, properties of EPSs can vary. In the present study, the antiviral activity of EPSs from *Tetraselmis suecica* and *Porphyridium cruentum* cultured under autotrophic and heterotrophic conditions has been evaluated *in vitro* against Viral Haemorrhagic Septicaemia Virus (VHSV), an important pathogen in fish farming. Results showed that EPSs from both species have anti-VHSV activity. *T. suecica* EPSs from autotrophic cultures showed the strongest effect, since both, adsorption and post-adsorption phases of the VHSV multiplication cycle were affected. In contrast, both, autotrophic and heterotrophic *P. cruentum* EPSs showed anti-VHSV activity only after the adsorption phase. These results pave the way to use these EPSs to fight VHSV infections, and animate to evaluate the EPS antiviral activity against other viral pathogens relevant to the aquaculture industry.

Introduction

The control of infectious diseases in aquaculture facilities, is one of the biotechnological applications of microalgae-derived products (Charoonnart et al., 2018). In particular, polysaccharides have been specially considered, since they may have different physicochemical properties, and show immunomodulatory, anti-inflammatory, antifungal, antibacterial, antiviral, antioxidant, and antitumoral activity (reviewed in Severo et al., 2022; Laroche, 2022).

Microalgae may contain important amounts of polysaccharides that can be classified into structural cell wall polysaccharides; intracellular storage polysaccharides; and matrix polysaccharides, also called exopolysaccharides (EPSs), which can be attached to the cell wall or be excreted (Mohammed et al. 2021; Laroche 2022). EPSs are especially interesting, since, as part of the adaptation mechanisms of microalgae to their environment, their production yield and composition, and, therefore, their properties, depend on culture conditions (Costa et al. 2021). In this regard, some microalgae can be cultured in autotrophic, mixotrophic, and heterotrophic conditions (Brennan and Owende, 2010). The heterotrophic culture is particularly advantageous, since it does not require light, and microalgae growing under this condition show higher biomass yield, growth rate, and increased synthesis of metabolites with biotechnological application (Pérez-García et al., 2011; Morales-Sánchez et al., 2015; 2017).

Tetraselmis suecica (*Chlorophyta*), and *Porphyridium cruentum* (*Rodophyta*), are two microalgal species that are normally cultured under autotrophic conditions in order to be used for different purposes. *T. suecica* is a marine green microalga widely used in aquaculture as live food for rotifers and copepods or artemia in hatcheries (Day et al., 1991). It has antibacterial activity (Austin and Day, 1990; Austin et al., 1992), probiotic properties (Irianto and Austin, 2002) and it has been proposed as a source of vitamin E for humans (Carballo-Cárdenas et al., 2003). *P. cruentum* is a red microalga encapsulated by an envelope

of sulphated polysaccharides, which have been widely used for important biotechnological applications (Rodriguez-Concepcion et al. 2018; Setyaningsih et al. 2020; Casas-Arrojo et al., 2021; Ginzberg et al., 2000; Patil et al., 2007). Recently, the heterotrophic culture of both species has been established, and important differences regarding production, composition, and cytotoxicity on mammalian cell lines have been recorded between EPSs from autotrophic and heterotrophic cultures (Parra-Riofrío et al. 2020; 2021).

In the present study, we look deeper into the properties of autotrophic and heterotrophic EPSs from *T. suecica* and *P. cruentum*. Particularly, the antiviral activity of these polysaccharides has been evaluated against Viral Haemorrhagic Septicaemia Virus (VHSV), *Novirhabdovirus piscine* species, *Novirhabdovirus* genus, *Rhabdoviridae* family, which is an enveloped virus with a non-segmented, negative-sense, single-stranded RNA genome. VHSV is responsible for an important disease affecting a notably broad range of marine and freshwater fish species, including several of the most relevant species in fish farming, such as rainbow trout, *Oncorhynchus mykiss* (He et al., 2021).

Material And Methods

2.1. Culture of T. suecica and P. cruentum

T. suecica and *P. cruentum* were obtained from the microalga collection (strain codes nº UMA-260920 and UMA-200997) of the Institute of Biotechnology and Blue Development (IBYDA) (Malaga, Spain).

T. suecica and *P. cruentum* were autotrophically cultured in F/2 (Guillard, 1975) and Vonshak media (Vonshak, 1988), respectively. The photoperiod was 12 h light (irradiance 165 µmol photons m-2 s-1) and 12 h darkness (12:12). For heterotrophic cultures, F/2 medium was supplemented with 5 g L^{-1}glucose and 100 units L^{-1} penicillin-10 mg L^{-1} streptomycin (Biowest); and Vonshak medium was supplemented with 3 g L^{-1} glucose, 100 units L^{-1} penicillin, 10 mg L^{-1} streptomycin (Biowest) and 0.25 µg L^{-1} amphotericin b (Biowest). Heterotrophic cultures were maintained in darkness. All cultures were maintained in agitation with an air bubbling system, at 21 ºC, and 35‰ or 27‰ salinity, for *T. suecica* and *P. cruentum* respectively, until stationary phase.

2.3. Exopolysaccharide extraction

Cells from cultures at stationary phase were removed by centrifugation (4500 rpm for 5 min at 4°C), and phenols were removed from supernatants by polyvinylpyrrolidone (Sigma) precipitation and the subsequent centrifugation (4500 rpm, 5 min, at 4°C) before EPS extraction. Total EPSs were precipitated with ethanol 1/1 (v/v) for 24 h (Sun et al., 2014), and acid EPSs were precipitated with 2% (w/v) N-cetyl pyridinium bromide (Cetavlon) (Sigma) for 24 h (Morris et al., 2000).

Precipitated EPSs were collected by centrifugation (4500 rpm, 5 min, at 4°C), and dissolved in 4 M NaCl (10 mL, Sigma). Once dissolved, ethanol was added (1/1, v/v) and the mixture was stored at 4°C for 24 h. After centrifugation at 4500 rpm, 5 min, at 4°C, the pellet containing polysaccharides and salts was

dialyzed (Sigma) against 0.5 M NaCl overnight at 4°C. Dialyzed EPSs were centrifuged (4500 rpm, 5 min, at 4°C), and washed with absolute ethanol. Finally, acid and total EPSs from both culture conditions (per triplicate, n = 3) were stored at -80°C and subsequently freeze-dried at -50°C.

2.5. Cell culture and virus propagation

The RTG-2 cell line, derived from gonad tissue of rainbow trout (Wolf and Quimby, 1962), was cultured at 20 ◦C with Leibovitz L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), 4 mM L-glutamine (Biowest), and 100 units L^{-1} penicillin-10 mg L^{-1} streptomycin (Sigma) (growth medium).

The VHSV reference strain DK-F1 (genotype I, obtained from rainbow trout) was propagated and titrated on RTG-2 cells cultured at 15 ◦C with L-15 medium supplemented with 2% FBS, 4 mM L-glutamine and 100 units L^{-1} penicillin-10 mg L^{-1} streptomycin (maintenance medium). Titration was carried out according to the 50% tissue culture infective dose ($TCID_{50}$) method (Reed and Muench, 1938).

2.6. Cytotoxicity assays

RTG-2 cells (2.5 x 10^4 cells/well) were seeded on 96-well plates and cultured in growth medium with different concentrations of each EPS, ranging from 19 to 1 x 10^4 µg mL^{-1}. Four replicas of each concentration were analysed. Non-treated cells were used as control. After 72-h incubation at 20°C, EPS cytotoxicity was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma)-reduction assay, according to Abdala-Díaz et al. (2019). Optical density (OD) was measured at 550 nm using a Whittaker Microplate Reader 2001. Relative cell viability was determined as the mean percentage of viable cells compared to untreated cells, and the medium inhibitory concentration (IC_{50}) of each EPS was also calculated. Measurements were carried out in independent experiments in triplicate. EPSs from each trophic condition in each species showing the lowest cytotoxicity were selected for the antiviral activity assays.

2.7. Antiviral activity assays

To evaluate the effect of EPSs on VHSV adsorption to host cells, RTG-2 cells (6 x 10^5 cells/well) were seeded on 24-well plates and incubated at 20 ºC for 24 h. Afterwards, cells were washed with PBS, and EPSs (100 µg mL^{-1}) and VHSV (0.2 multiplicity of infection, MOI) were subsequently added to the cells in L-15 medium supplemented with 4 mM L-glutamine and 100 units L^{-1} penicillin-10 mg L^{-1} streptomycin (inoculation medium). After 1 h at 15 ºC (viral adsorption time), the inoculation medium was removed, cells were washed with PBS, and maintenance medium was added. These cells were incubated at 15º C, and they were collected for viral genome quantification at 0, 12, 24, and 36 h post-inoculation (p.i.). In addition, cellular supernatants were also sampled to quantify the extracellular infective viral particles by the TCID50 method. Three wells per condition were analysed. Non-treated cells, non-infected cells treated with EPSs, and infected cells without EPS treatment, were also included.

To evaluate the post-adsorption effect of EPSs, RTG-2 cells were seeded as described above, incubated for 24 h, washed with PBS, and infected with VHSV (0.2 MOI) in inoculation medium for 1 h at 15º C. After viral adsorption, the inoculation medium was removed, cells were washed with PBS, and maintenance medium with 100 µg mL^{-1} EPS was immediately added. At 0, 12, 24, and 36 h p.i., cells were collected for viral genome quantification, and extracellular viral particles were titrated by the TCID50 method. Replicates and controls were as described above.

Cellular RNA was extracted using the EZNA total RNA Kit, and treated with RNase-free DNase I (Sigma-Aldrich) before cDNA synthesis, which was carried out with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Resulting cDNA was stored at -20 oC until used.

Viral genome was quantified by absolute real-time PCR. Amplifications were conducted with the LightCycler 96 Thermocycler and the Fast Start Essential DNA Green Master Mix, using cDNA generated from 50 ng of RNA. Amplification conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. Primers used are shown in Table 1. Three replicates of each PCR were performed. Serial dilutions of the pGemT easy vector (Thermo) containing the VHSV nucleoprotein gene sequence, were used as reference standard curve.

Table 1
Primers used in this study

	Name	Primer sequence (5´-3´)	Accession No.	Reference
VHSV	VHSV-F1	AAGGCCCTCTATGCGTTCATC	AJ23396	Alvarez-Torres
n gene	VHSV-R1	GGTGAACAACCCAATCATGGT		et al., 2013

2.8. Statistical analyses

All data were statistically analyzed by one-way analysis of variance (ANOVA) using the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Differences of $P < 0.05$ were considered statistically significant.

Results

3.1. Cytotoxicity of T. suecica and *P. cruentum EPSs*

In order to determine the EPSs cytotoxicity on RTG-2 cells, IC_{50} values were calculated (Table 2). Regarding autotrophic *T. suecica* cultures, total EPSs showed higher toxicity (19x) than acid EPSs, whereas acid EPSs from heterotrophic cultures were more cytotoxic (13x) than total EPSs. Concerning *P. cruentum*, a similar result was observed: total EPSs from autotrophic cultures were more cytotoxic on RTG-2 cells than acid EPSs (3.5x); and acid EPSs from heterotrophic cultures were more toxic (37x) than total EPSs. Based on these results, acid EPSs from autotrophic cultures and total EPSs from heterotrophic cultures from both microalgal species were selected for antiviral assays.

Table 2
IC_{50} values on RTG-2 cells of total and acid EPSs extracted from autotrophic and heterotrophic cultures of *T. suecica* and *P. cruentum*. Asterisks indicate EPSs selected for antiviral activity evaluation.

	Culture	EPS	IC_{50} µg mL^{-1}
T. suecica	Autotrophic	Total	240 ± 2.0
		Acid	4550 ± 0.7*
	Heterotrophic	Total	9020 ± 1.4*
		Acid	680 ± 0.1
P. cruentum	Autotrophic	Total	720 ± 0.1
		Acid	2500 ± 0.5*
	Heterotrophic	Total	1080 ± 0.2*
		Acid	29 ± 0.1

3.2. Anti-VHSV activity of T. suecica EPSs

The presence of EPSs from autotrophic cultures of *T. suecica* during viral adsorption time resulted in the complete inhibition of viral genome replication and, consequently, the inhibition of infective viral particle production (Fig. 1A, B). In contrast, EPS obtained from heterotrophic cultures of this microalga caused a reduction in the number of copies of the viral genome, compared with untreated cells, only at 36 h p.i., whereas the infective viral particles production decreased at 24 and 36 h p.i. (Fig. 1C, D).

Similar results were obtained when EPSs were added after viral adsorption and maintained during all the incubation period (post-adsorption assay). Specifically, a complete inhibition of viral genome replication and infective viral particles production was recorded in cells exposed to EPSs obtained from autotrophic cultures (Fig. 2A, B), with only a slight increase in the viral titre recorded at 36 h p.i. Regarding EPSs from heterotrophic cultures, no differences between control and EPS-exposed cells were recorded concerning viral genome, whereas a significant decrease in infective viral particles production was recorded at 24 and 36 h p.i. in EPS-exposed cells compared to control cells (Fig. 2C, D).

3.3. Anti-VHSV activity of P. cruentum EPSs

If *P. cruentum* EPSs from both, autotrophic and heterotrophic cultures, were present during VHSV adsorption, final (36 h p.i.) amounts of genome copies were similar to that recorded in control untreated cells (Fig. 3A, C). Actually, a lower viral genome replication was only detected at 24 and 12 h p.i. for autotrophic and heterotrophic EPSs, respectively, (Fig. 3A, C). However, both, heterotrophic and autotrophic EPSs, caused a decreased production of infective viral particles at 24 and 36 h p.i. (Fig. 3B,

D). On the contrary, the addition of *P. cruentum* EPSs after VHSV adsorption resulted in the abolition of viral genome replication (Fig. 4A, C) and infective viral particles production (Fig. 4B, D).

Discussion

Antiviral activity is one of the most attractive applications of microalgal EPSs. Actually, an increasing number of studies proving the antiviral activity of these molecules is being published in last years (Shi et al., 2017; Pereira, 2018; Pereira and Critchley, 2020; Carbone et al., 2021). Most of these studies report positive results against a great number of important human viral pathogens, such as herpes simplex virus, human cytomegalovirus, influenza A virus, human immunodeficiency virus, human papillomavirus, dengue virus, rhinovirus, or even SARS-coV-2. In contrast, the EPS effect against fish viruses has been scarcely evaluated (Fabregas et al., 1999; Katharios et al., 2005; Jyotsna et al., 2021). In the present study, a clear antiviral effect of *T. suecica* and *P. cruentum* EPSs has been demonstrated against VHSV, an important pathogen for the aquaculture industry, supporting EPSs as major molecules in the biotechnological use of microalgae, and EPS antiviral activity as one of their most valuable applications in aquaculture.

Only one out of the eight different EPSs evaluated in this study was cytotoxic on RTG-2 cells: acid EPSs from heterotrophic cultures of *P. cruentum* (IC_{50} values < 100 µg mL^{-1}). The lack of cytotoxicity of EPSs extracted from *T. suecica* and *P. cruentum* cultured in autotrophic and heterotrophic conditions has also been proven in mammalian cells; however, those substances are highly cytotoxic on tumoral mammalian cells, thus making these molecules attractive candidates for antitumor therapies (Parra-Riofrío et al., 2020; 2021). Interestingly, in all cases, important differences between acid and total EPS IC_{50} values were recorded (Table 2), showing acid EPSs from autotrophic cultures and total EPSs from heterotrophic cultures higher IC_{50} values. Differences in the effect of each EPS are probably due to their specific composition, which is quite different regarding content of proteins, carbohydrates, lipids and pigments, as well as monosaccharide composition (Parra-Riofrío et al., 2020; 2021).

The antiviral activity of the selected EPSs was evaluated by two parameters: viral genome replication, estimated by viral genome quantification in cells, and infective viral particle production, estimated by viral titres in supernatants. As expected, when viral genome replication was completely abolished, no infective viral particles were produced. This has been reported for *T. suecica* EPSs from autotrophic cultures in both, adsorption and post-adsorption assays (Figs. 1A, B; Figs. 2A, B), and for *P. cruentum* EPSs (heterotrophic and autotrophic) acting after viral adsorption (Fig. 4). When viral genome replication was only partially inhibited, as in the adsorption assay of *T. suecica* EPSs from heterotrophic cultures, a reduced production of infective viral particles was observed (Fig. 1C, D). Interestingly, even when viral genome replication was not affected (post-adsorption assay of *T. suecica* EPSs from heterotrophic cultures (Fig. 2C, D) the production of infective viral particles was reduced, which indicates that EPSs are interfering viral assembling and/or release. In the adsorption assays of *P. cruentum* EPSs (Fig. 3), genome amount was similar at 36 h p.i., but lower at earlier time points, thus suggesting a delay in viral

genome replication that could be responsible for the lower production of infective viral particles (Fig. 3B, D).

Microalgal EPSs may exert their antiviral activity by interfering with different stages of the viral infection and replication process (Shi et al., 2017; Chen and Huang, 2018; Carbone et al., 2021). Regarding their anti-adsorption activity, EPSs can directly interact with viral particles, and/or can bind to cell receptors, competing with viral particles (Harden et al., 2009; Shi et al., 2017). The most evident effect on VHSV adsorption was recorded for *T. suecica* EPSs from autotrophic cultures (Fig. 1A, B). Furthermore, these EPSs also showed a strong post-adsorption effect (Fig. 2A, B). Although further experiments should be conducted to determine the mechanism or mechanisms involved in the observed effects, *T. suecica* EPSs from autotrophic cultures showed an attractive potential application to fight VHSV infections in aquaculture.

EPSs from heterotrophic cultures of *T. suecica* also showed adsorption and post-adsorption effects, although at a lower level than EPSs from autotrophic cultures (Figs. 1 and 2). In contrast, *P. cruentum* EPSs showed a much stronger post-adsorption effect, and no differences between EPSs from autotrophic or heterotrophic cultures were recorded (Figs. 3 and 4). These differences can be due to the specific composition of each EPS type. It has been proposed that post-adsorption antiviral effect of microalgal EPSs involves EPS interaction with cell surface receptors, which would activate the innate immune response, and/or would interfere viral genome uncoating, transcription, translation, and/or capsid assembly (Shi et al., 2017; Carbone et al., 2021).

The anti-VHSV activity of extracts from both species, *T. suecica*, and *P. cruentum*, has been previously tested (Fábregas et al., 1999). In contrast with our results, no antiviral activity was recorded for *T. suecica* extracts; however, *P. cruentum* extracts showed anti-VHSV adsorption activity. The cell line used (epithelioma papulosum cyprinid, EPC cells); the experimental design (adsorption assays, after overnight incubation of algal extracts with VHSV at 4 ºC); the parameter used to detect antiviral activity (number of infected cells foci detected by immunostaining); and, more importantly, the low purification level of microalga extracts, could be responsible for the different results recorded. In previous studies, *P. cruentum* extracts had shown activity against African swine fever virus, Herpes simplex viruses (type 1 and 2) and Varicella zoster virus (Fábregas et al., 1999; Huleihel et al., 2001). In contrast, to the best of our knowledge, this is the first report on *T. suecica* antiviral activity, although previous studies had demonstrated its activity against bacterial fish and crustacean pathogens (Austin and Day, 1990; Austin et al., 1992).

In summary, a clear antiviral activity of EPSs from *T. suecica* and *P. cruentum* against VHSV has been demonstrated. *T. suecica* EPSs from autotrophic cultures showed the strongest effect, as both, adsorption and post-adsorption phases of VHSV multiplication cycle in RTG-2 cells were affected. These results pave the way to use microalgal EPSs to fight viral diseases in aquaculture. To that end, it would be necessary to conduct studies focused on (i) the identification of EPS bioactive molecules; (ii) disclosing

the underlying mechanisms of the antiviral activity; (iii) *in vivo* assays; and (iv) evaluation of EPS antiviral activity against other viral pathogens relevant for the aquaculture industry.

Declarations

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Conflict of interest

The authors have not disclosed any competing interests.

Author contributions statement

Conceptualization, E.U.-T., R.T.A-D., E.G.-R., M.C.A. and J.B.; methodology, G.P.-R. and P.M.G.; formal analysis, G.P.-R. and P.M.G.; Supervision, E.G.-R., M.C.A. and J.B.; writing—original draft preparation, J.B.; writing—review and editing, G.P.-R., P.M.G., R.T.A-D., E.G.-R., and M.C.A.; funding acquisition and project administration, E.U.-T., R.T.A-D., E.G.-R., M.C.A. and J.B. All authors reviewed the manuscript.

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Figures

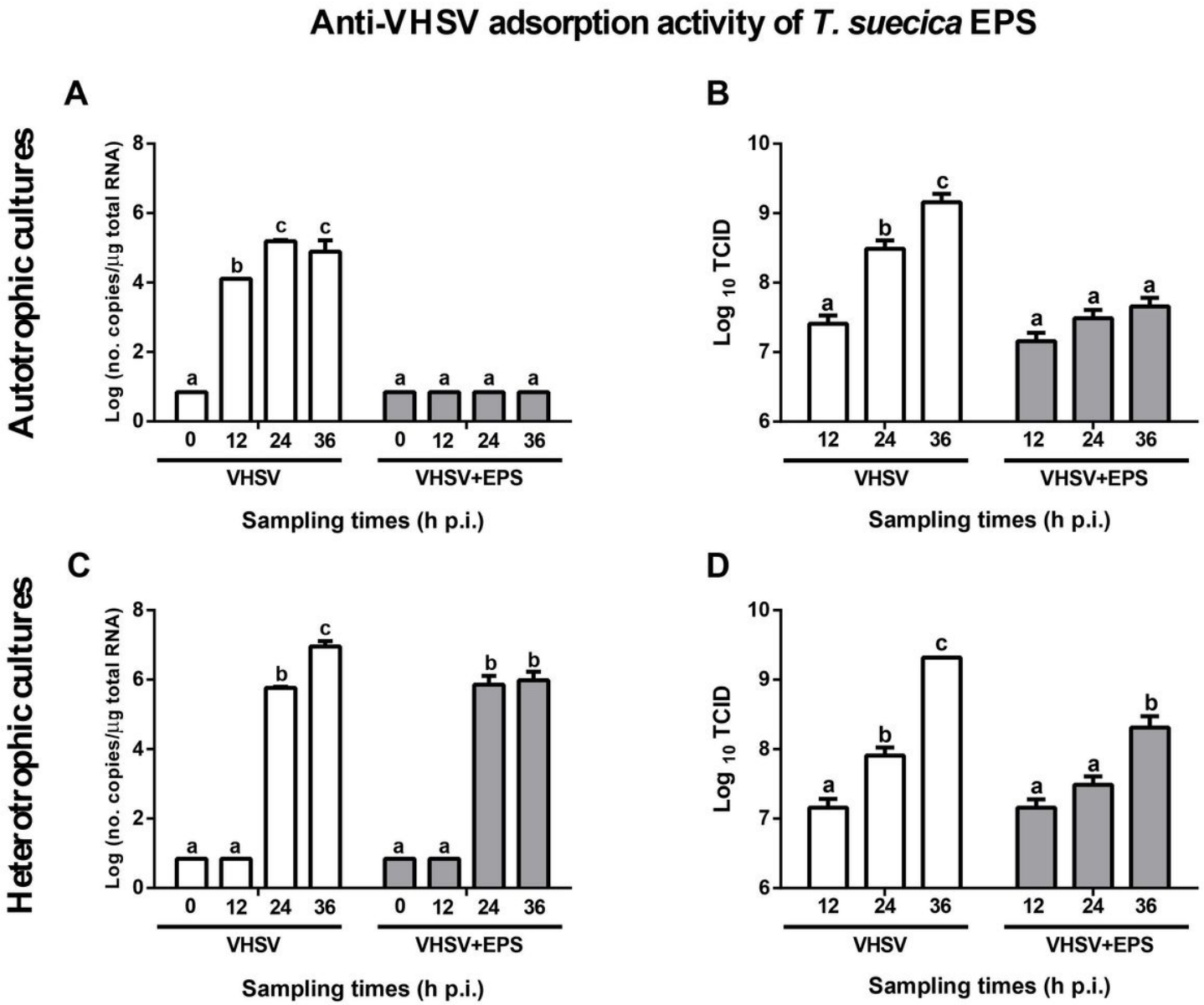

Figure 1

Anti-VHSV adsorption activity of autotrophic (A and B) and heterotrophic (C and D) EPSs from *T. suecica* at 12, 24 and 36 h p.i.. A, C) Viral genome quantification by absolute real time qPCR. B, D) Extracellular infective viral particles ($TCID_{50}$ mL^{-1}). Different letters indicate significant differences between groups ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3).

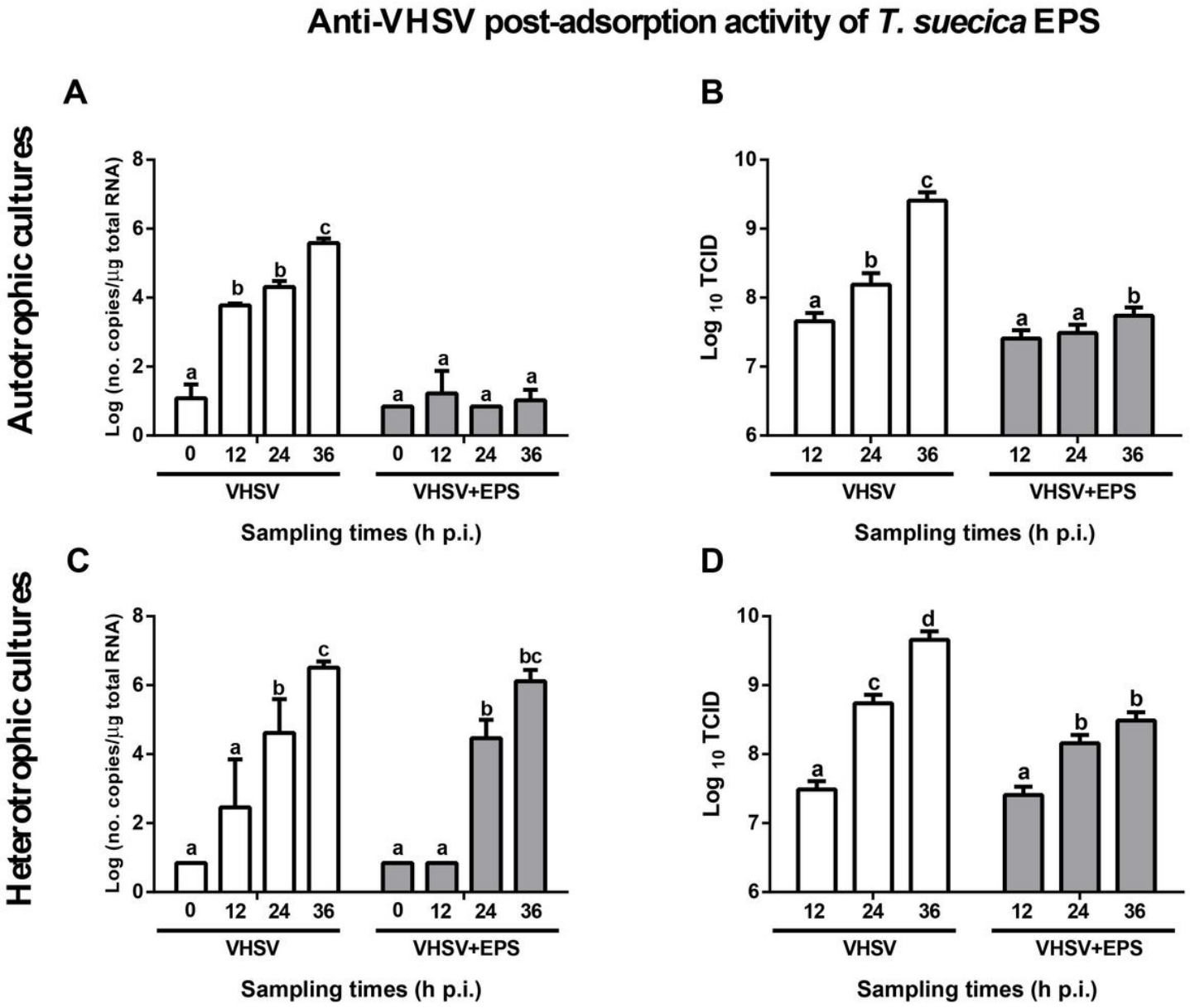

Figure 2

Anti-VHSV post-adsorption activity of autotrophic (A and B) and heterotrophic (C and D) EPSs from *T. suecica* at 12, 24 and 36 h p.i.. A, C) Viral genome quantification by absolute real time qPCR. B, D) Extracellular infective viral particles ($TCID_{50}$ mL^{-1}). Different letters indicate significant differences between groups ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3).

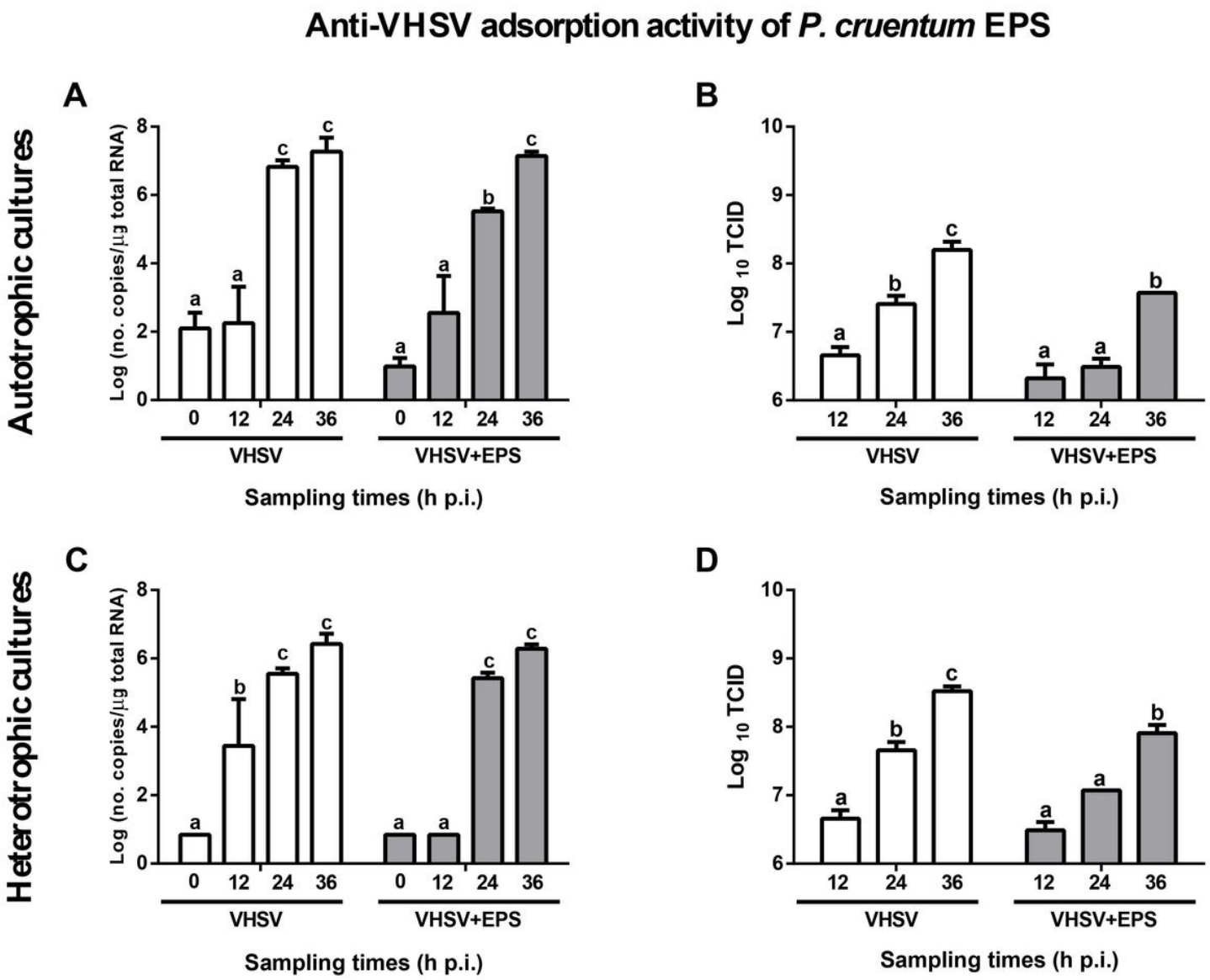

Figure 3

Anti-VHSV adsorption activity of autotrophic (A and B) and heterotrophic (C and D) EPSs from *P. cruentum* at 12, 24 and 36 h p.i.. A, C) Viral genome quantification by absolute real time qPCR. B, D) Extracellular infective viral particles ($TCID_{50}$ mL^{-1}). Different letters indicate significant differences between groups ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3).

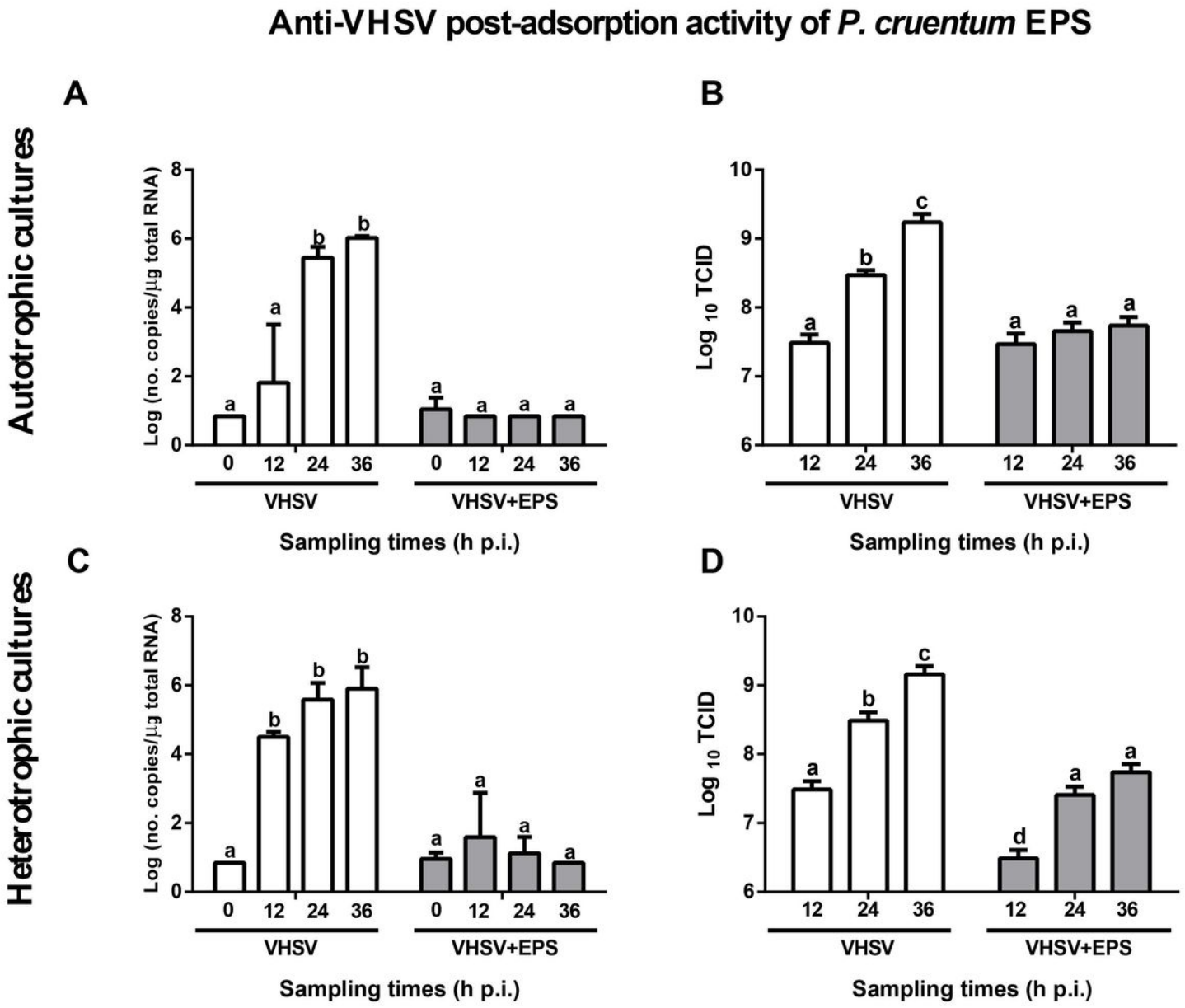

Figure 4

Anti-VHSV post-adsorption activity of autotrophic (A and B) and heterotrophic (C and D) EPSs from *P. cruentum* at 12, 24 and 36 h p.i.. A, C) Viral genome quantification by absolute real time qPCR. B, D) Extracellular infective viral particles ($TCID_{50}$ mL^{-1}). Different letters indicate significant differences between groups ($p < 0.05$). Results are mean ± standard deviation (SD) (n = 3).