Dextrans produced by lactic acid bacteria exhibit antiviral and immunomodulatory activity against salmonid viruses

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Abbreviations: BF-2, bluegill fry; CC_{50}, dextran concentration provoking 50% inhibition of cell growth; CDM, defined medium containing 0.8% glucose; CDMS, CDM without glucose and supplemented with 0.8% sucrose; $TCID_{50}$ mL^{-1}, virus titer infecting 50% of the cell culture; DOSY, diffusion ordered spectroscopy; $EC_{50,}$ dextran concentration reducing the viral cytopathic effect to 50% of control value; EPC, epithelioma papulosum cyprinid; EPS, exopolysaccharide; FBS, fetal bovine serum; HK, head kidney; IHNV, infectious hematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; IR, infrared, LAB, lactic acid bacteria; MM, L15 medium with 2% FBS; MOI, multiplicity of infection; MRS, Man Rogosa Sharpe broth; MRSS, MRS medium supplemented with 2% sucrose instead of glucose; NMR, nuclear magnetic resonance; PBS, Phosphate buffered saline; qPCR, quantitative real time PCR polymerase change reaction; RT-qPCR, reverse transcription qPCR; SI, selective index.

Abstract

Viral infections in the aquaculture of salmonids can lead to high mortality and substantial economic losses. Thus, there is industrial interest in new molecules active against these viruses. Here we describe the production, purification, and the physicochemical and structural characterization of high molecular weight dextrans synthesized by *Lactobacillus sakei* MN1 and *Leuconostoc mesenteroides* RTF10. The purified dextrans, and commercial dextrans with molecular weights ranging from 10-2000 kDa, were assayed in infected BF-2 and EPC fish cell-line monolayers for antiviral activity. Only T2000 and dextrans from MN1and RTF1 had significant antiviral activity. This was similar to results obtained against infectious pancreatic necrosis virus. However the dextran from MN1 showed ten-fold higher activity against hematopoietic necrosis virus than T2000. *In viv*o assays using the MN1 polymer confirmed the *in vitro* results and revealed immunomodulatory activity. These results together with the high levels of dextran production (2 g L^{-1}) by *Lb. sakei* MN1, indicate the compounds potential utility as an antiviral agent in aquaculture.

Keywords: Lactic acid bacteria, exopolysaccharide, dextran, antiviral, immunostimulants, salmonid virus, IPNV, IHNV.

1. Introduction

Salmonid fish viruses cause considerable losses to the aquaculture industry worldwide. Aquabirnaviruses such as the infectious pancreatic necrosis virus (IPNV), and novirhabdoviruses such as infectious hematopoietic necrosis virus (IHNV), cause acute diseases of rainbow trout (*Onchorhynchus mykiss*) and several species of salmon. The mortalities are inversely proportional to the age of the fish and survivors remain carriers of the virus for long periods. IPNV was the first virus isolated from fish, in 1960, and is considered the most widely distributed aquatic virus (Rodríguez Saint-Jean, Borrego, & Pérez-Prieto, 2003). IHNV was first observed on the west coast of the USA in cultivated sockeye salmon (*Oncorhynchus nerka*) in 1953, and has been also isolated in Japan and several European and Asian countries (Bootland & Leong, 1999).

The use of existing antivirals against fish viruses has not been investigated in depth due to high product costs and potential toxicity. Alternative antivirals have been proposed such as olive leaf extract (Micol et al., 2005) and milk casein (Rodríguez Saint-Jean et al., 2012). Prevention and control of disease is currently approached in fish farming by the use of immunostimulants such as β-glucans from yeast, fungi or bacterial origin, alone or supplemented with other compounds (for example: mannose, LPS or vitamin C) (Ringø, Olsen, González-Vecino, Wadsworth, & Song, 2012). Thus, alternative antiviral molecules that could block replicating virus in infected individuals are of great interest in the battle against virus infections. Ideally, these new drugs should induce strong and specific inhibition of virus replication without affecting cellular processes. Among them, polysaccharides besides β-glucans, could have antiviral activity and this possibility deserves to be investigated.

Many lactic acid bacteria (LAB) produce exopolysaccharides (EPS) with different compositions, structures, molecular masses and conformations. These compounds have many industrial applications. High molecular weight polysaccharides are widely used in the food industry as texturizers, stabilizers, viscosifiers, and emulsifiers among other applications (Ruas-Madiedo & de los Reyes-Gavilán, 2005; Werning et al., 2012). Dextrans are used in the production of fine chemicals such as plasma substitutes and Sephadex®. In addition, they could also have an attractive application in the control of viruses in aquaculture due to the harmless nature of these compounds.

Dextrans are composed of repeating units that contain only D-glucopyranose and the linkages in the main chain are α-1,6. In addition, they may have side-chain branches that involve other α-linkages different from that in the main chain (α-1,2; α-1,3 or α-1,4). These polymers are synthesized and secreted by LAB and they are produced by strains belonging to the genera *Lactobacillus*, *Leuconostoc, Streptococcus* and *Weisella* (Leemhuis et al., 2013). The dextran most widely used in industry contains 95% α-1,6 and 5% α-1,3 linkages and is synthesized by *Leuconostoc mesenteroides* NRRL B-512F. This was the first industrial dextran produced for different medical and biotechnological applications (Naessens, Cerdobbel, Soetaert, & Vandamme, 2005).

In the present study, we have characterized as dextran the EPS produced by *Lb. sakei* MN1, evaluated the *in vitro* and *in vivo* antiviral activity of the purified EPS against IHNV and IPNV and investigated its mechanism of action.

2. Materials and Methods

2.1. Bacterial strains and growth media

The two LAB strains used in this work were isolated from meat products at the Department of Food Biotechnology, Institute of Agrochemistry and Food Technology (C.S.I.C., Valencia, Spain).They were characterized and identified as *Lb. sakei* MN1 and *Lc. mesenteroides* RTF10 by 16S rRNA coding gene sequencing (accession number in GenBank: KJ161303 and KJ161304, respectively) at Secugen (Madrid, Spain). The strains were grown at 30 ºC without agitation in defined CDM medium (Poolman, Hellingwerf, & Konings, 1987) supplemented with 0.8% sucrose (CDMS) instead of glucose or in Man Rogosa Sharpe broth (MRS, De Man, Rogosa, & Sharpe, 1960) supplemented with 2% sucrose (MRSS) instead of glucose. Strains were kept in MRS supplemented with 20% (v/v) glycerol for long-term storage and maintained at −80 ºC.

2.2. Quantification, purification and characterization of EPS

EPS production was determined in liquid media. Strains were grown in MRSS to an OD_{600nm} of 2. Cells were washed, resuspended in the same volume of fresh MRSS media and diluted 1:100 in CDMS medium. Batch fermentations without pH control were performed, in triplicate, for the two strains and they were grown in 100 mL screw-capped flasks for 10 h at 30 ºC. Cultures were sampled every hour to monitor growth and EPS production. For EPS quantification, bacterial cells were removed from the growth medium by centrifugation at 9,300 x *g* for 10 min at 4 ºC. The EPS was precipitated from the supernatants by the addition of three volumes of cold absolute ethanol and incubation for 12 hours at -20 ºC. After centrifugation at 9,300 x *g* for 20 min at 4 ºC, the precipitate was washed twice with 80% ethanol and sedimented by

centrifugation. The EPS pellet was air dried, then suspended in distilled water and heated for 10 min at 30 ºC. Total sugar content was determined by the phenol/sulphuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Determinations were performed in duplicate. EPS large scale production and purification was performed as previously described (Notararigo et al., 2013). Briefly, LAB strains were grown in MRS supplemented with 2% sucrose to an OD_{600nm} of 1.0 for *L. mesenteroides* RTF10 or of 2.5 for *L. sakei* MN1. Cells were removed from culture supernatant by centrifugation and EPS were recovered by precipitation with an equal volume of ethanol at 96% and centrifugation. Then, the EPS were resuspended in water and were purified by dialysis using a membrane (12-14 kDa cutoff) and chromatographic fractionation through a Sepharose CL-6B column. After this purification the yield of the EPS were 1.2 g L^{-1} for MN1 and 0.8 g L^{-1} for RTF10 strains and the levels of contaminant DNA, RNA and proteins were below the detection levels (0.5 µg mL^{-1}, 20 ng mL^{-1} and 1 µg mL^{-1}) by use of specific fluorescent staining kits and the Qubit® 2.0 fluorometric detection methods (Molecular probes). EPS structure was analyzed by different approaches that included monosaccharide composition, phosphate content, methylation analysis and infrared (IR) spectra using the same protocols described by Notararigo et al. (2013). For nuclear magnetic resonance (NMR) analysis, EPS (15 mg) were dissolved in deuterated water (0.5 mL). The magnitude of the molecular mass of the EPS was estimated by diffusion ordered spectroscopy (DOSY). The standard Bruker DOSY protocol was used at 298 K with the ledbpg2s pulse sequence on an Avance 500 MHz spectrometer. Thirty-two 1D ^{1}H spectra were collected with a gradient duration of δ=4 ms and an echo delay of Δ=400 ms. Samples

of commercially available dextrans with different molecular weights were used to build the calibration curve.

2.3. Antiviral activity assay against IHNV and IPNV

2.3.1. Fish cell lines and viruses

Experiments were performed using the bluegill fry (BF-2) fish cell line from *Lepomis macrochirus* (ATCC, CRL 1681) and epithelioma papulosum cyprinid (EPC) cells from *Pimephales promelas* (ATCC, CRL 2872). Both cell lines were grown in Leibovitz medium (L15; Gibco, Invitrogen, Barcelona, Spain) supplemented with 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS; Gibco, Invitrogen) at 25 °C. Maintenance medium was based on the L15 medium with only 2% FBS (MM). For antiviral activity assay, the VR714 strain of IHNV and the Sp serotype of IPNV were obtained from the American Type Culture Collection (ATCC VR714 and ATCC VR1318, respectively). For virus propagation the protocols and methods were performed as previously described (Rodríguez Saint-Jean & Pérez-Prieto, 2006). Their viral titer was determined by assaying the dose that infected 50% of the cell culture ($TCID_{50}$ mL^{-1}) (Pérez-Prieto et al., 2001). BF-2 cells were used as the host of the IPNV and EPC cells for IHNV.

2.3.2. In vitro cytotoxicity assay

Purified dextrans were tested for cytotoxicity by evaluating the cell viability and proliferation using a colorimetric assay as previously described (Rodríguez Saint-Jean & Pérez-Prieto, 2006). BF-2 and EPC fish cell line monolayers were incubated in the presence of different dextran concentrations for 3 (BF-2) or 7 (EPC) days at 20 °C. Cells were fixed and stained using 1% crystal violet in 20% ethanol, then the cells were washed with water three times, air dried and the absorbance was measured in a

131 microplate reader (BIORAD, Hercules, CA, USA) at 590 nm. Results were expressed as
132 percentage of surviving cells, where 100% represents the dye absorbance of the control
133 cells incubated in MM.
134 *2.3.3. Antiviral activity assay*
135 Antiviral activity was determined as previously described (Rodríguez Saint-Jean &
136 Pérez-Prieto, 2006). Cells were seeded in 96-well culture plates and for each treatment a
137 mock-infected control was included (toxicity controls) and some cell wells were left
138 untreated and infected (virus control). When the control virus wells affected more than
139 80% of the cell monolayer, the cells were processed by the colorimetric assay described
140 above. Inhibition of the viral cytopathic effect on the dextran treated cells was measured
141 as percentages, where 100% inhibition represents the average of the dye absorbance
142 value of non-infected cells treated in the same way as infected cells.
143 *2.3.4. Virus yield reduction assay*
144 The assay was performed according to Rodríguez et al. (2012). Briefly, sets of four
145 cultures growing in 48 well plates were infected with IPNV or IHNV at a multiplicity of
146 infection (MOI) of 0.1 and, after 1 h adsorption at 20 ºC or 15 °C respectively, the
147 inoculum was removed, the monolayers washed with phosphate buffered saline (PBS),
148 and various concentrations of EPS were added. At 3 or 5 days after infection with IPNV
149 or IHNV, the cultures were frozen and thawed three times to lyse the cells, centrifuged
150 (3000 x *g*, 10 min), and the supernatants stored at -70 °C until they were used for viral
151 titration. The total yield (intracellular and extracellular) of infectious virus was
152 determined by infecting four parallel well of cell monolayers with ten-fold dilutions of
153 the samples.
154 *2.3.5. Addition time assay*

155 BF-2 or EPC cells (5 x 10^4 cells per well) were seeded in 48-well plates and
156 pretreated with dextrans (1000 µg mL^{-1}) for 30 min before, simultaneously to or 1 h
157 after infection with IPNV or IHNV at a MOI of 0.1. The cultures (in quadruplicate)
158 were incubated for 3 days after infection, and the cells were then recovered by scraping.
159 Following three consecutive freeze–thaw cycles, the supernatants of the clarified lysates
160 were collected, and the amount of infectious virus was determined by $TCID_{50}$ assay.
161 For a direct viricidal test, samples were first mixed with an equal volume of the
162 IHNV or IPNV suspension (at a final dose of 100 $TCID_{50}$ mL^{-1}) and incubated for 1 h at
163 15 °C (IHNV) or 20 °C (IPNV). The amount of infectious virus was determined by
164 $TCID_{50}$ assay.

165 *2.4.* In vivo *assay*

166 *2.4.1. Experimental fish*

167 The rainbow trout fry, mean body weight 1.5 g, were purchased from a commercial
168 fish farm with no history of viral disease. The fish were kept under a 12/12 h light/dark
169 regime at 15 °C in 350 L closed tanks with re-circulating water (Living Stream, Frigid
170 Units Inc, Ohio) at the Centro de Investigaciones Biológicas (Madrid, Spain). The fish
171 were fed (3% of their body weight) twice a day with a diet of commercial pellets and
172 they were maintained as described previously Pérez-Prieto et al. (2001).The trout were
173 anaesthetized by immersion in 50 µg mL^{-1} buffered tricaine methanesulphonate (MS-
174 222; Sigma) prior to handling. The experiments described comply with the Guidelines
175 of the European Union Council (86/609/EU) for the use of laboratory animals.

176 To evaluate disease resistance, the fish were acclimatized in the laboratory for 2
177 weeks before experiment. The trout were distributed in seven groups (n = 20) of treated

and control fish. Group 1 and 2: treated with 10 µg per fish of EPS diluted in saline solution during three day. Group 3 and 4 treated with 50 µg per fish of EPS diluted in saline solution during three days. EPS administration was performed using an automatic pipette with a 20 µL tip which was introduced into the mouth of each fish, supporting the tip end at the entrance of the oesophagus. Group 5, 6 and 7 were maintained during 3 days without treatment.

On the third day of treatment the fish belonging to groups 1, 3 and 5 were infected by immersion in IHNV (5×10^5 $TCID_{50}$ mL^{-1}) as previously described (De Las Heras, Rodríguez Saint-Jean, & Pérez-Prieto, 2010). The fish of groups 2, 4 and 6 were infected by immersion in IPNV (5×10^5 $TCID_{50}$ mL^{-1}). Normal control (Groups 7), IHNV control (Group 5) and IPNV control (Group 6) underwent the same stress handling without the antiviral compound. Water-quality parameters were maintained at optimum levels, and the culturing conditions in all tanks were equivalent. All groups were kept under observation for 30 days to record clinical signs and mortality rate. Two replicas of the trial were performed.

Relative percentage survival was calculated 30 days after challenge according to the following formula 1-[% mortality of fish given vaccine/mortality of virus control fish] × 100.

2.4.2. Gene expression analysis

Two fish groups were used for *in vivo* stimulation. Two groups were injected intraperitoneally with either EPS from MN1 (50 µg mL^{-1}) or poly I:C (25 µg mL^{-1}) and other group was injected with 100 µL PBS per fish. At days 1, 3, 7 and 15 after treatment, fish (n=3) from each group were randomly sampled and killed with MS-222. Anterior kidney tissue was removed and processed for RNA extraction to evaluate the

expression of selected genes (IFN-1 and IFN-γ) by reverse transcription quantitative real time PCR analysis (RT-qPCR).

Total RNA extraction was carried out using Trizol (Invitrogen) according to the manufacturer's instructions. A Nanodrop ND 1000 spectrophotometer was employed to analyze RNA concentration and purity. All samples were DNase treated to remove any contaminating DNA.

cDNA was synthesized using the SuperScript III First – Strand Synthesis System (Invitrogen, Madrid, Spain) with random primers in a 20 µL final reaction volume. The qPCR were performed by using the SYBR® green method, in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories, Madrid, Spain). The qPCR amplifications were carried out in 96-well plates by mixing 1 µL of four-fold diluted cDNA, 12.5 µL of 2 x concentrated iQ SYBR Green Super Mix (Bio Rad), 0.3 µM forward primer and 0.3 µM of reverse primer in 25 µL reaction volume for each sample. The thermal profile was 10 min at 95 ºC followed by 40 amplification cycles of 10 s at 95 ºC, 1 min at 60 ºC and a dissociation cycle (1 min at 95 ºC and 1 min at 60 ºC). After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification. All qPCR reactions were performed in triplicate and for each mRNA, gene expression values were normalized to that of the endogenous control (elongation factor 1-α). The folds change relative to control expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), where $\Delta\Delta Ct_{gene} = \Delta Ct_{gene} - \text{mean } \Delta Ct_{control}$.

The qPCR primers used were IFN1F (5´-AAAACTGTTTGATGGGAATATGAAA-3´) and IFN1R (5´-CGTTTCAGTCTCCTCTCAGGTT-3´) for IFN-1, IFNR (5´-CTGAAAGTCCACTATAAGATCTCCA-3´) and IFNγR (5´-

226 CCCTGGACTGTGGTGTCAC-3´) for IFN-γ and, EF-F (5´-
227 GATCCAGAAGGAGGTCACCA-3´) and EF-R (5´-TTACGTTCGACCTTCCATCC-
228 3´) for elongation factor 1-α.

229 Statistical Analysis

230 Data are presented as mean ± standard deviation. Factorial ANOVAs were run to
231 determine if the differential expression gene differed between the dextran treated and
232 untreated groups. The p values <0.05 were considered significant.

3. Results

3.1. EPS production and characterization

The rate of EPS production by the two strains in defined CDMS medium supplemented with sucrose was monitored during 10 h and is shown in Fig. 1. Both strains synthesized EPS during the exponential phase of growth. Maximum production was 2 g L^{-1} ± 0.1 g L^{-1} for *Lb. sakei* MN1 (EPS-LS) and 1.4 g L^{-1} ± 0.05 g L^{-1} for *Lc. mesenteroides* RTF10 (EPS-LM). Both EPS were purified for further analysis. The IR spectra of the two EPS preparations, and the commercial T2000 dextran, were essentially the same (Fig. 2A and Notararigo et al., 2013) and they were typical of carbohydrates, with an absorption band around 850 cm^{-1} and another at 916 cm^{-1} both attributable to α-anomers. Observation of the signals defined further some chemical characteristics of these compounds. In the methylation studies the two EPS gave the partially methylated and partially acetylated derivates of a (1-6) glucan with approximately 6% substitution, at positions *O*-3, by side chains composed of a single residue of glucose (Fig. 2B and Notararigo et al., 2013). The data from methylation analysis and IR spectra defined the polymer as a dextran-type polysaccharide with the structure depicted in Fig. 2C. According to the DOSY analysis the two bacterial dextrans, EPS-LS and EPS-LM, had similar molecular masses (data not shown). When they were compared with the commercial dextran T2000 (EPS-LS and T2000 analysis is shown in Fig. 3), a great difference in molecular mass was detected, with a logarithmic diffusion coefficient of -10.9 for T2000 and -12.3 for the two EPS tested. T2000 is a high purity dextran fraction commercialized with the highest average molecular weight ($2x10^6$ Da) and molecular weight distribution. Thus, EPS-LS and EPS-LM have an average molecular weight > $2x10^6$ Da. Due to this very high

molecular weight a more precise determination could not be obtained by NMR nor by chromatographic analysis, since appropriate commercial molecular markers do not exist.

3.2. Potential as antiviral agent against salmonid fish viruses

The cytotoxicity of the purified EPS-LS and EPS-LM and T2000 dextrans was assayed against BF-2 and EPC fish cell line monolayers prior to the *in vitro* analysis of antiviral activity against IPNV and IHNV. The three dextrans proved not to be toxic up to the maximum concentration tested in this work (5000 µg mL^{-1}). Once the non-toxic character of the dextrans had been ascertained, the inhibition of IPNV and IHNV fish viral infections was determined using a straightforward assay based on the reduction of the cytopathic viral effect. The three dextrans showed antiviral activity (Table 1) though differential behavior among them was observed. Selective index (SI) was calculated by the ratio CC_{50}/EC_{50}. At all concentrations assayed, the three dextrans showed a similar antiviral activity against IPNV virus (results not shown). A concentration of 1000 (for EPS-LS and EPS-LM) or 3000 (commercial dextran T2000) µg mL^{-1} was required to obtain a 50% inhibition (Table 1).

EPS-LS and EPS-LM showed a similar antiviral activity against the virus IHNV in the range of concentrations tested, in contrast to T2000 that showed a very low activity (Fig. 1S). In addition, EPS-LS showed the highest antiviral activity resulting in a 50% inhibition at a concentration of 500 µg mL^{-1} (Table 1). A ten-fold higher concentration (5000 µg mL^{-1}) of T2000 was required to obtain the same effect (Table 1). EPS-LS inhibits IHNV replication in EPC cells with a selective index of more than 10. The more potent inhibition of EPS-LS and EPS-LM against IHNV could be explained by their molecular weight than T2000 (Fig. 3). Therefore, the antiviral action of commercial

281 dextrans with a molecular weight ranging from 10-500 kDa (T10, T50, T70 and T500)
282 was also analyzed (Table 1 and Fig. S1). No antiviral activity of T10, T50 and T7
283 against IPNV and IHNV was detected. Similarly, this reduction of the molecular weight
284 of the commercial dextrans from 2000 kDa to 500 kDa almost abolished their influence
285 on IPNV infection increasing the EC_{50} from 3000 to >5000 (Table 1) and provoking a
286 reduction of infectivity from 50% for T2000 to 25% for T500 at a concentration of the
287 dextrans of 1 μg mL^{-1} (Fig. 1S).

288 *3.3. Inhibitory effect on IPNV and IHNV infection by dextrans*

289 The influence of the polymers on the viruses' replication in cell cultures was
290 analyzed by the viral yield reduction assay. For IPNV, a significant reduction in virus
291 yield of 2.5 or 2 logarithmic units was recorded in the BF-2 cells treated with either
292 EPS-LS or EPS-LM at the highest concentration tested (1000 μg mL^{-1}). The EPS-LS
293 was the only dextran whose addition at 250 μg mL^{-1} resulted in a decrease of viral
294 infectivity (1 logarithmic unit). By contrast, for T2000 no significant influence in
295 infective viral titre was observed at any concentration tested (Fig. 4A).

296 In the case of IHNV, a 4, 2 or 1.5 logarithmic units reduction in virus yield was
297 detected in the EPC cells treated with EPS-LS, EPS-LM or T2000 at 1000 μg mL^{-1}. At
298 lower doses (from 100 to 500 μg mL^{-1}), the two EPS also reduced viral infectivity (in
299 the range of 1.5 to 3.5 logarithmic units) with a greater effect of EPS-LS, whereas in
300 this dose-range T2000 only produced a slight effect (0.5 logarithmic unit reduction) at
301 500 μg mL^{-1} (Fig. 4B).

302 *3.4. Influence of addition time of dextran on yield of IPNV and IHNV after infection*

303 To identify at which stage of the virus cycle the dextrans could impair viral
304 infection, addition-time assays were performed using dextrans at a concentration of
305 1000 µg mL^{-1} (Fig. 5).

306 In BF-2 cells infected with IPNV, the addition of any of the three dextrans, 30 min
307 prior to infection, elicited some antiviral activity (1 logarithmic unit for both EPS and
308 0.5 for T2000). Higher antiviral activity was observed when the dextrans were added at
309 the time of infection or 60 min later. In these cases, in the BF-2 cells treated with EPS-
310 LS, EPS-LM or T2000, a decrease of approximately 2.5, 1.5 or 1 logarithmic units in
311 virus yield was detected (Fig. 5A).

312 Similar behaviour was observed in the EPC cells infected with IHNV (Fig. 5B).
313 However, in this case, the reduction in virus yield provoked by EPS-LS addition was
314 much higher when this dextran was added at the time of exposure or 60 min after
315 infection, with a reduction of viral infectivity of approximately 5 logarithmic units.

316 *3.5. The effect of EPS-LS on the survival of IPNV- and IHNV-infected rainbow trout*

317 Since EPS-LS showed the highest antiviral activity *in vitro* (Table 1 and Figs. 4 and
318 5), its influence at 10 µg mL^{-1} and 50 µg mL^{-1} was analyzed *in vivo* (Fig. 6). Dextran-
319 untreated fry trout infected with IPNV began to show characteristic signs of infectious
320 IPNV diseases from day 2 after infection (Fig. 6A). On this day mortality began in this
321 group, increasing in a stepwise manner through days 4, 7, 11 and 13, and finally
322 stopping around day 16. In infected fish treated with 10 µg mL^{-1} of EPS-LS, signs of the
323 disease were observed on day 6 and deaths registered were 1 fish on day 7, 1 fish on day
324 12 and 1 fish on day 19. In fish pretreated with EPS-LS at 50 µg mL^{-1} the signs of the
325 disease were observed on day 6 and deaths registered were only 1 fish on day 7. The
326 non infected control fish showed a normal behaviour with a survival of 96% at the end

of the experiment, whereas the accumulated mortality in untreated fish infected with IPNV (positive control) were 53.3 %. In fish pretreated with 10 or 50 μg mL^{-1} of EPS-LS the accumulative mortality was only of 20% and 6.6% respectively. Thus, EPS-LS at 50 μg mL^{-1} increased the survival from 46.7% (untreated control) to 87.5%.

Dextran-untreated fry infected with IHNV began to show characteristic signs of infectious IHNV disease from day 5 after infection (Fig. 6B). On day 6 mortality began in this group reaching a 33% on days 12, a 60% on days 17 and a final value of 73% on day 30. In infected fish treated with 10 μg mL^{-1} EPS-LS, signs of the disease started on day 11 and deaths registered were 1 fish on days 11, 13, 15, 19 and 2 fish on day 21. In infected fish treated with 50 μg mL^{-1} EPS-LS, signs of the disease were observed on day 11 and deaths registered were 1 fish, on days 12, 15 and 21. The accumulated mortality in untreated fish infected with IHNV was 73.3%, whereas in fish pretreated with 10 and 50 μg mL^{-1} was 40% and 20%, respectively. Thus, pretreatment with 50 μg mL^{-1} EPS-LS raised the fish survival from 22.7% to 72.7%.

3.6. Kinetics of differential expression of interferon genes in trout head kidney

To investigate the potential immunomodulatory activity of EPS-LS, expression of the selected genes (IFN-1 and IFN-γ) was analyzed by RT-qPCR in trout head kidney (HK) at different times after treatment with the dextran. Because HK was the main trout internal immunological responsive organ, it was chosen to study the kinetics of expression of IFN.

The fold increases observed in HK of treated group in comparison to their corresponding untreated control group is shown in Fig. 7 for IFN-1 and IFN-γ expression. In the case of IFN-1, the immunostimulant of innate response poly I:C was also tested as a control, and an up-regulation was observed 1 day after treatment, which

decreased to almost control levels on day 2 (Fig. 7A). The EPS-LS produced an up-regulation of the expression of both glycoproteins. As expected, accordance with the involvement of the IFN tested, either an innate or adaptive immunological response to EPS-LS was detected at different time points. The maximum value for IFN-1 transcript was observed 3 days after treatment, reaching a level of 27.8-fold over the untreated control, higher than that induced by poly I:C (5.8-fold). Up-regulation mediated by EPS-LS remained high at day 7 and decreased almost to control levels at day 15. By contrast, and as expected for adaptive immune response, the IFN-γ mRNA levels only increased drastically at day 15 after treatment with EPS-LS. Thus, these results revealed that the bacterial dextran immunomodulates trout *in vivo*.

4. Discussion

The early events in the replication cycle of some types of viruses are relevant to the design of antiviral compounds. However, the potential to induce immune responses is also relevant in fish, and could be important in delaying the progression of a viral infection. Thus, there is an increasing interest in the evaluation of glucans as immuno-modulators, feed additives or adjuvants for vaccines in fish aquaculture. Studies have focused mostly on β-glucan activity against bacteria (Ai et al., 2007) or virus (Kim, Ke, & Zhang, 2009; Beaulaurier et al., 2012) but the mechanisms by which the immunity is enhanced is not well known. Glucan receptors have been reported in fish macrophages (Engstad & Robertsen, 1994; Ainsworth, 1994) and non-specific immunity by direct activation of macrophages has also been described (Sakai, 1999; Cook, Hayball, Hutchinson, Nowak, & Hayball, 2001). More recently other researchers have reported some results on the immunological activities of β-glucans. Falco et al. (2013) described up-regulation of TLR3 dependent genes and an increased antiviral activity of Mx protein, in common carp fed with β-glucan supplemented diets and stimulated with poly I:C.

In the present study we have evaluated another type of homopolysaccharide, namely, dextrans of bacterial origin. The EPS-LS and EPS-LM have shown *in vitro* and EPS-LS *in vivo* antiviral actions against fish viruses. Moreover, the *in vitro* experiments (Table 1, Figs. 4, 5 and 1S) indicated that the high molecular weight EPS LS and EPS-LM exhibit some properties or mechanisms that differ from those of known commercial dextrans. A more pronounced inhibitory effect of the dextrans was observed for IHNV (Fig. 4). Differences in the antiviral activity against IPNV and IHNV have also been detected for other natural compounds. In a previous work the antiviral activity of several

fractions of casein hydrolysates against fish viruses was examined (Rodríguez Saint-Jean et al., 2013). Significant inhibition of IHNV replication was observed in a dose-dependent manner, with the selective index ranging from 30 to 32, while the same molecules had only a moderate inhibitory activity against IPNV (antiviral index of around 3). This antiviral action is probably attributable to direct damage to the envelopes of the viruses. The IHNV is an enveloped virus that binds to a cell surface receptor through a G-protein attachment. Penetration into the cells is by endocytosis via coated pits. IPNV is a naked virus that enter the cells through vesicle uptake (Kuznar, Soler, Farias, & Espinoza, 1995). In a previous work (Rodríguez Saint-Jean et al., 2013), we postulated that some peptides from the casein protein hydrolysates may bind to cellular surfaces and compete for the virus receptors, blocking the membrane fusion. Here, a similar mechanism cannot be discarded. However, the fact that for both viruses the antiviral effect of the dextran was more prominent when treated with the polymer simultaneously with the virus or after viral infection (60 min) than when they were administrated 30 min prior to infection (Fig. 5), indicated that probably the dextrans act in a later step as effectors for the inhibition of the replication cycle. Further experiments will be required to reveal the mechanism of action of the EPS-LS and EPS-LM dextrans.

In vertebrates, interferons are secreted by host cells (including macrophages, lymphocytes, natural killer cells and fibroblasts), in response to recognition of double-stranded RNA intermediates of viruses such us IPNV and IHNV (Haller, Kochs, & Weber, 2006). Type I IFN is produced by leukocytes and fibroblasts as a response to viral infections. Type II IFN, represented by IFN-γ, is produced by natural killer cells and T-lymphocytes in response to IL-12, IL-18, mitogens or antigens (Robertsen, 2006),

409 with IFN-γ being a key activator of macrophages for increased killing of bacterial,
410 protozoal and viral pathogens. The mechanism of action of the dextrans is unknown, but
411 it is feasible that they may act as immunostimulants. This assumption is based in our
412 results, which revealed that the *in vivo* treatment of trout with EPS-LS α-glucan, in
413 addition to markedly decreasing mortality provoked by both IPNV and IHNV (Fig. 6),
414 significantly increased the expression of IFN-1 and IFN-γ (Fig. 7).

415 **Conclusions**

416 We have characterized two dextran-producing LAB isolated from fermented meat
417 products. Their purified dextrans have shown functional activity against salmonid
418 viruses, IPNV and IHNV. As far as we know, this work represents the first study on the
419 identification of antiviral activity of natural dextrans against fish viruses. Further studies
420 are required to determine the mechanisms responsible for the antiviral effect of these
421 biopolymers. Nevertheless, our results indicate that fish-food containing the high
422 molecular weight α-glucan from *Lb. sakei* MN1 would be effective in protection of
423 salmonids from viral infection. In addition, its stimulatory effect on the immune
424 response of fish through dietary supplements should have additional value for
425 commercial aquaculture.

426 **Acknowledgements**

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520 **Legends to the figures**

521 **Figure 1.** Exopolysaccharide production by *Lb. sakei* MN1 and *Lc. mesenteroides*
522 RTF10 in CDMS medium during 10 h growth. Growth (lines) and levels of
523 exopolysaccharide production (bars).

524 **Figure 2.** Physicochemical characterization of dextrans. (A) IR spectrum; (B) types of
525 bonds deduced by methylation analysis; (C) dextran structure.

526 **Figure 3**. Molecular mass analysis of dextrans using nuclear magnetic resonance
527 (DOSY). (A) comparison of the 2D-DOSY spectra EPS-LS and T2000 dextrans; (B)
528 superposition of the projections shown in (A) of the signals in the diffusion dimension.

529 **Figure 4.** Antiviral activity of different concentrations of dextrans against IPNV
530 infection of BF-2 cells (A), and IHNV infection of EPC cells (B). Symbols: black, dark
531 grey and white bars indicate treatment of infected cells with either EPS-LS, EPS-LM or
532 T2000 respectively. The results depicted are the average of three independent
533 experiments. Mean values and standard deviations are shown.

534 **Figure 5.** Influence of addition time on antiviral activity of dextrans against IPNV
535 infection of BF-2 cells (A), and IHNV infection of EPC cells (B). Symbols: black, dark
536 grey and white bars indicate treatment of infected cells with either EPS-LS, EPS-LM or
537 T2000 respectively. The results depicted are the average of three independent
538 experiments. Mean values and standard deviations are shown.

539 **Figure 6.** Antiviral protective activity of EPS-LS against IPNV (A) and IHNV (B)
540 infection of rainbow fry trout. Symbols: ♦, untreated infected trout; ▲ and ■, infected
541 trout treated with EPS-LS at 10 or 50 µg per fish.

542 **Figure 7.** Influence of EPS-LS on expression of IFN-1 (A) and IFN-γ. Levels of RNA
543 from head kidney of trout treated with either EPS-LS (black bars) or poly I:C (white

544 bars) were analyzed by RT-qPCR. The results depicted are the average of three
545 independent experiments. Mean values and standard deviations are shown.

Table 1. Dextran cytotoxicity and antiviral activity.

Dextran	[a]CC_{50} (µg mL^{-1})	IPNV		IHNV	
		[b]EC_{50} (µg mL^{-1})	[c]SI	[b]EC_{50} (µg mL^{-1})	[c]SI
EPS-LS	> 5000	1000	> 5	500	> 10
EPS-LM	> 5000	1000	> 5	750	> 6.7
T2000	> 5000	3000	> 1.7	5000	> 1
T500	> 5000	> 5000	> 1	> 5000	> 1
T70	> 5000	> 5000	> 1	> 5000	> 1
T10	> 5000	> 5000	> 1	> 5000	> 1

[a]CC_{50} is the dextran concentration at which 50% inhibition of cell growth is observed.

[b]EC_{50} is the dextran concentration needed to inhibit the viral cytopathic effect to 50% of control value.

[c]SI selective index is the CC_{50}/ EC_{50} ratio.

Figure(s)

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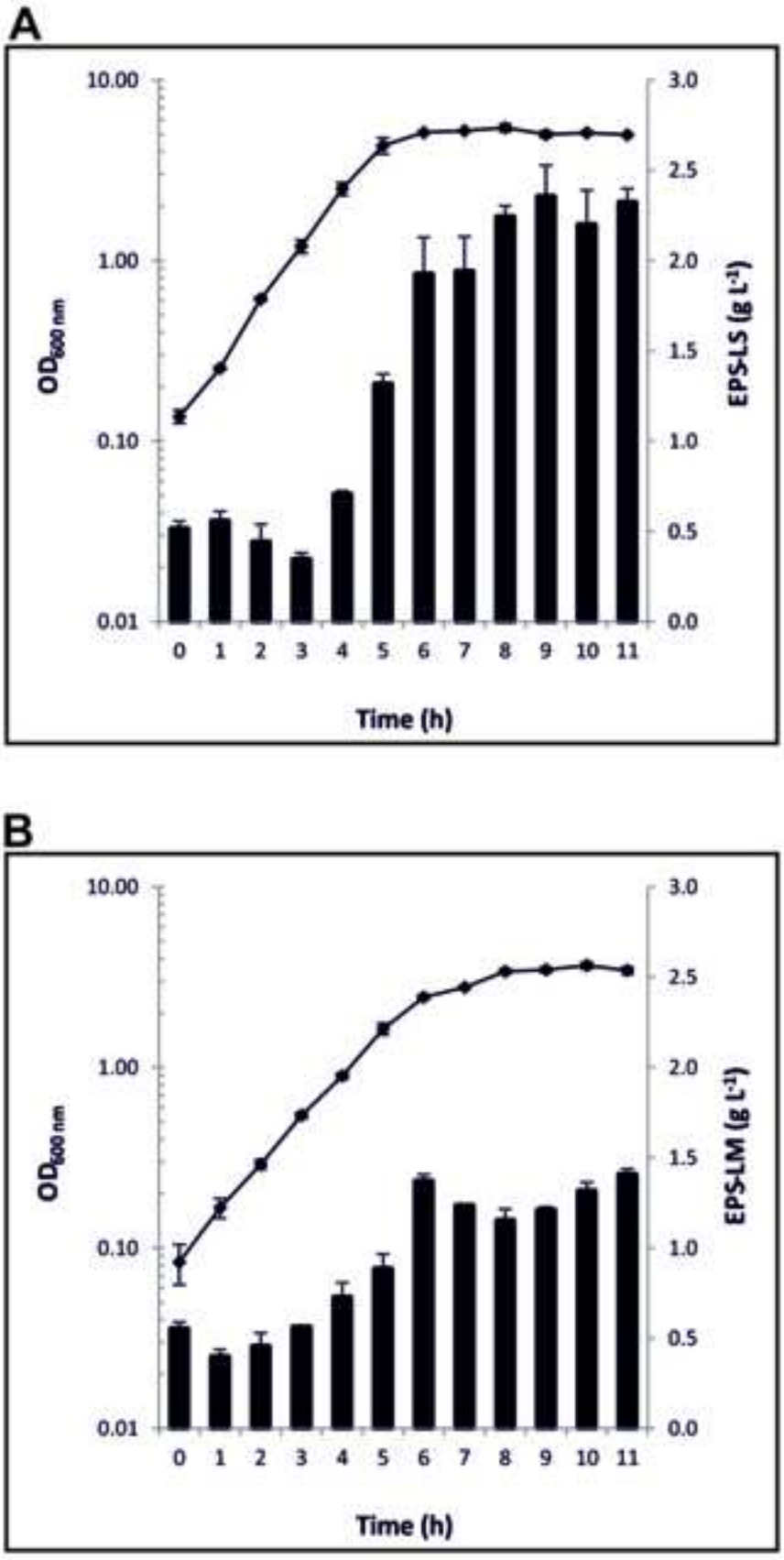

Fig. 1

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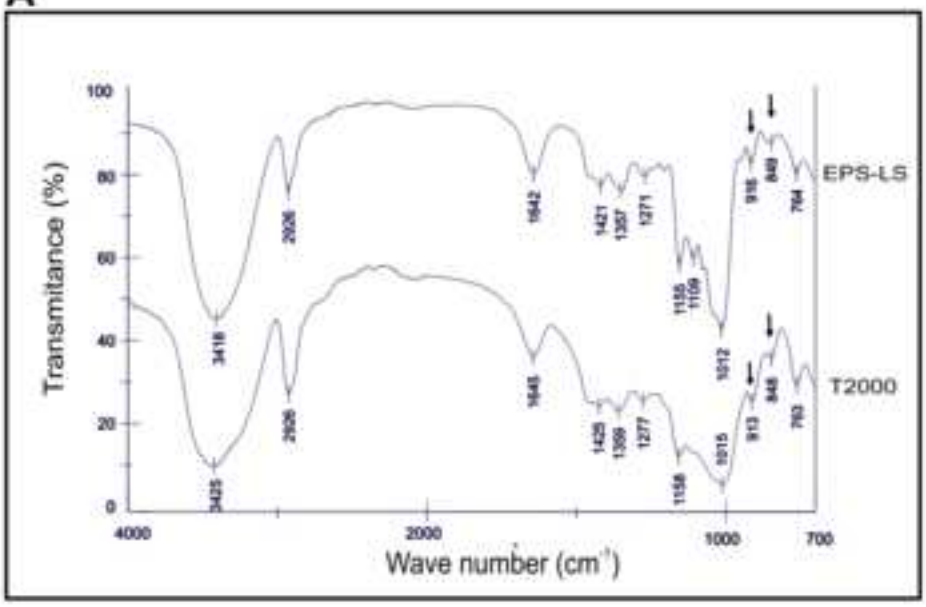

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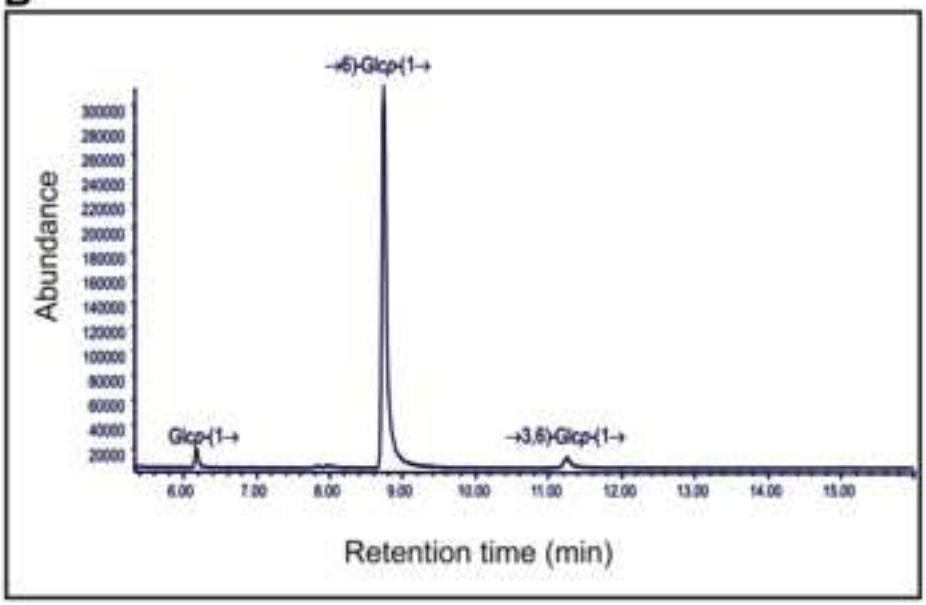

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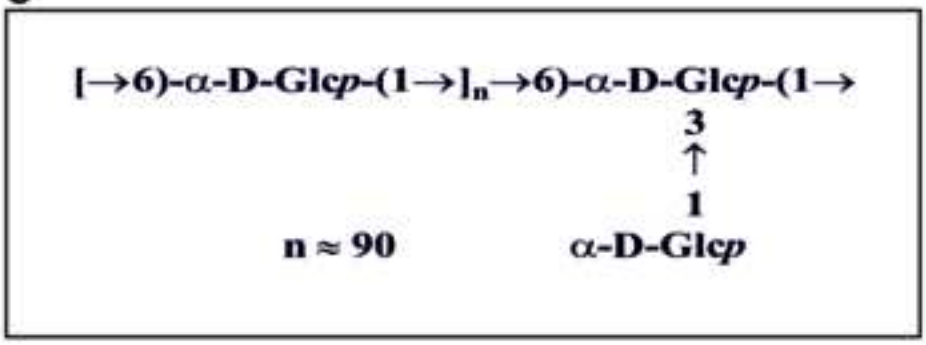

Fig. 2

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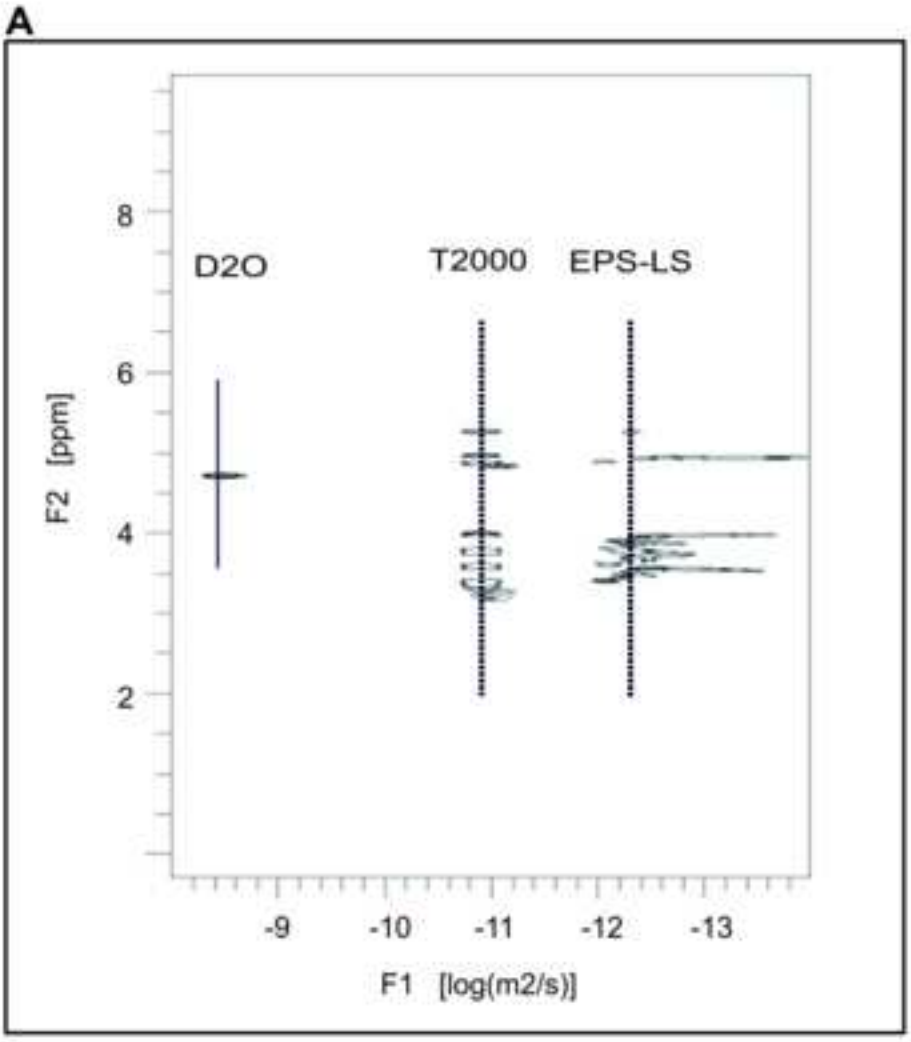

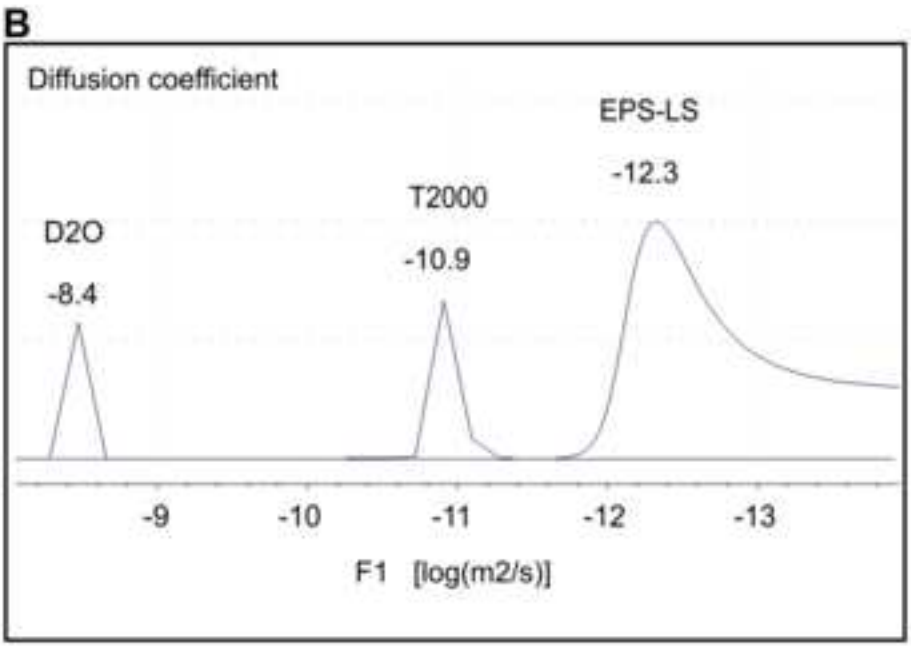

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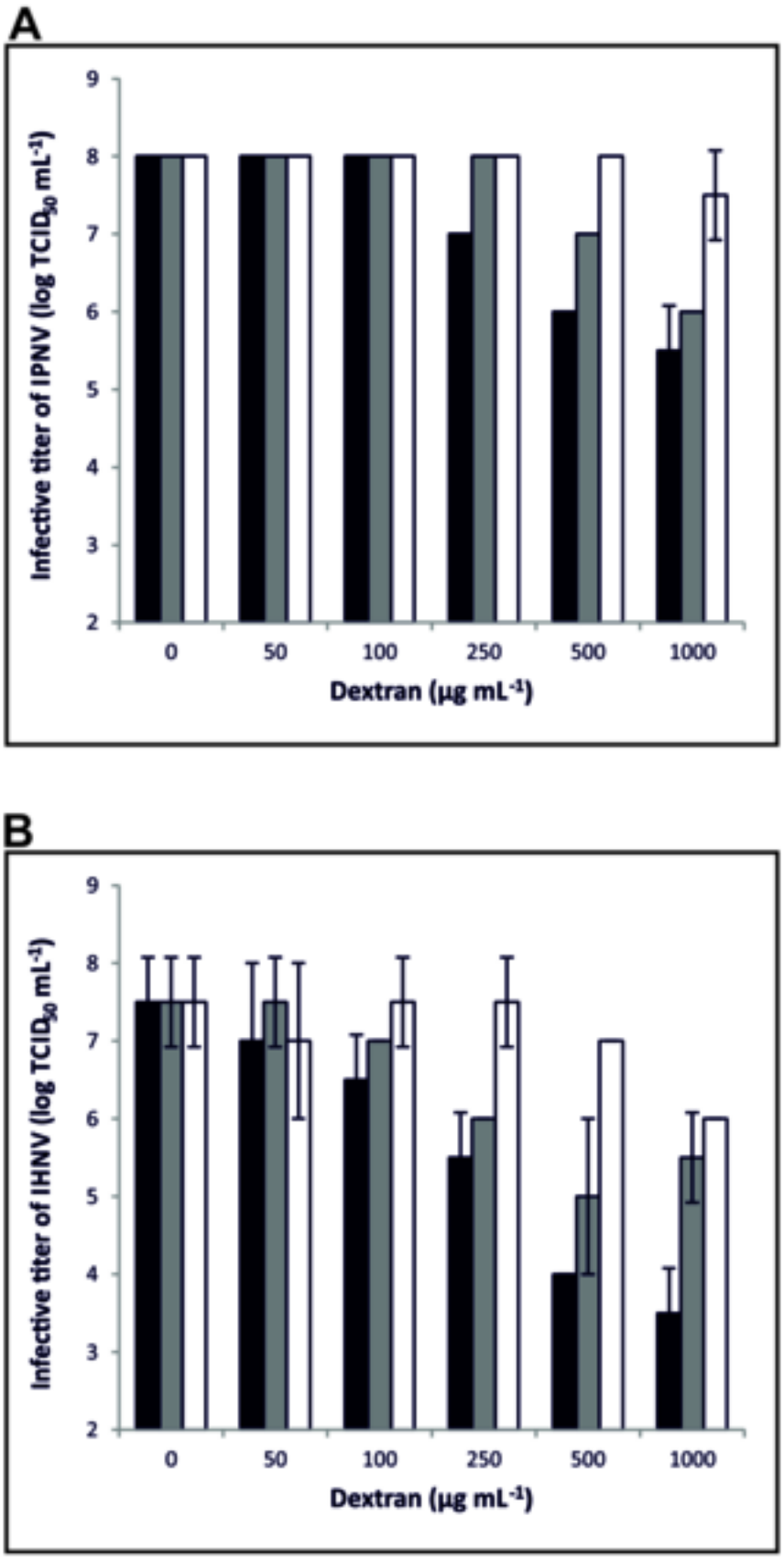

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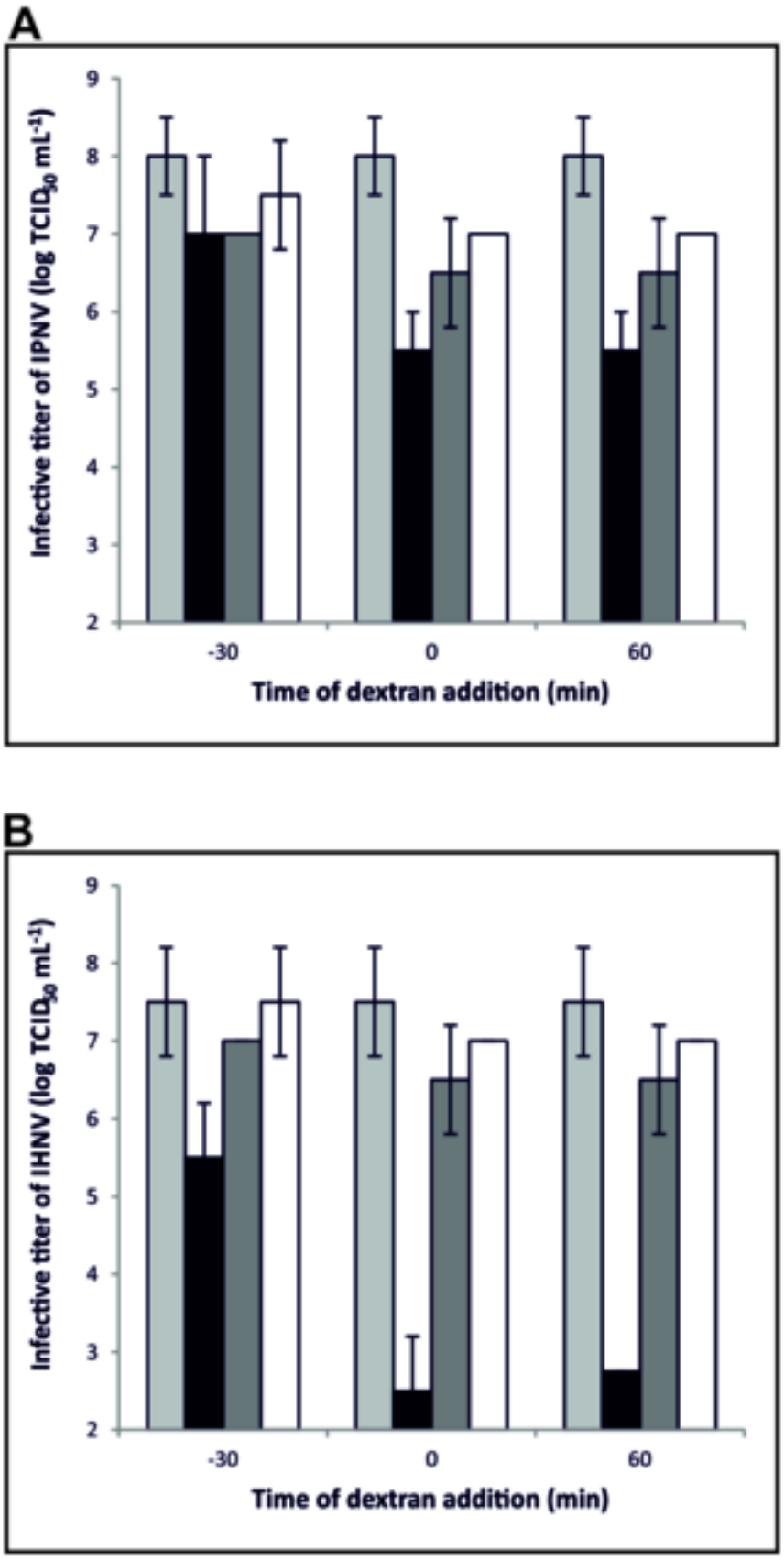

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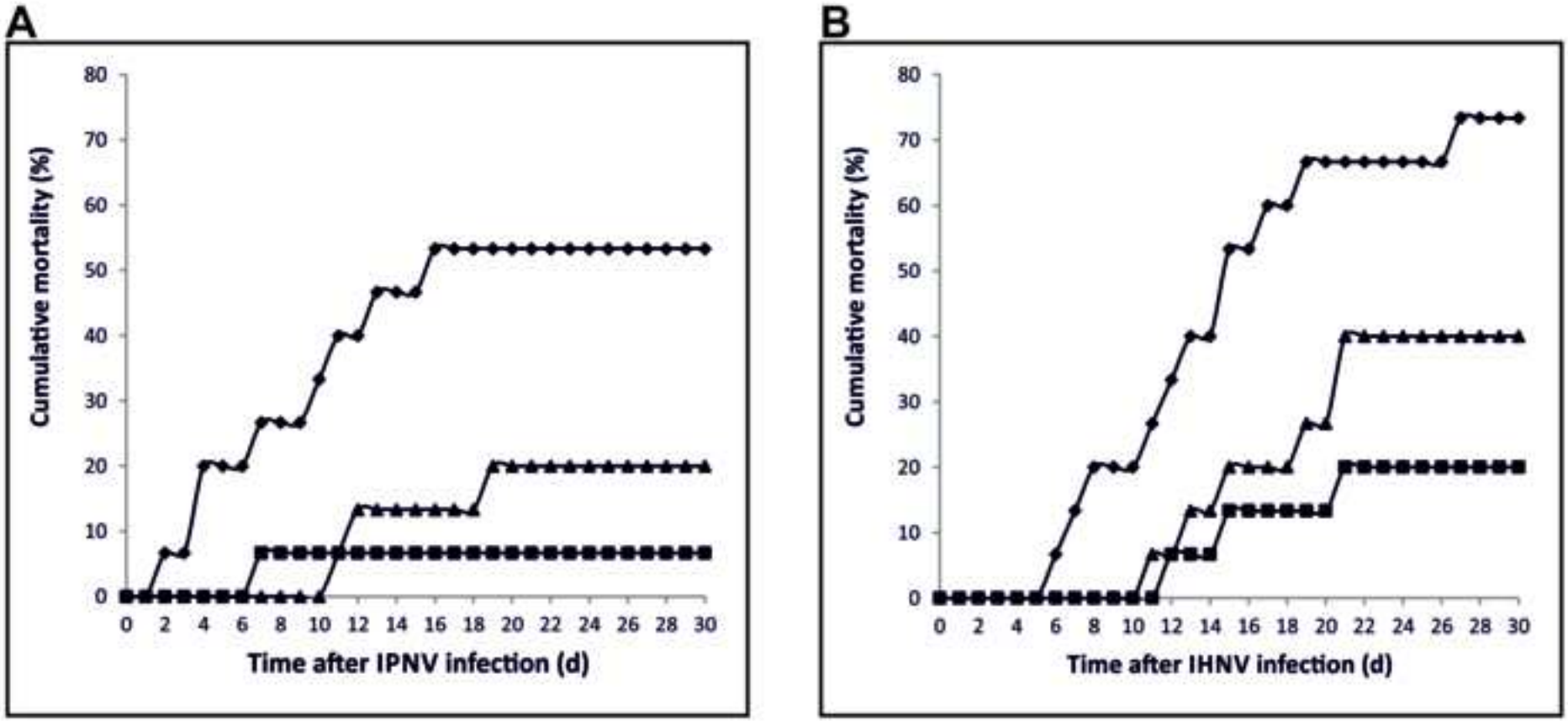

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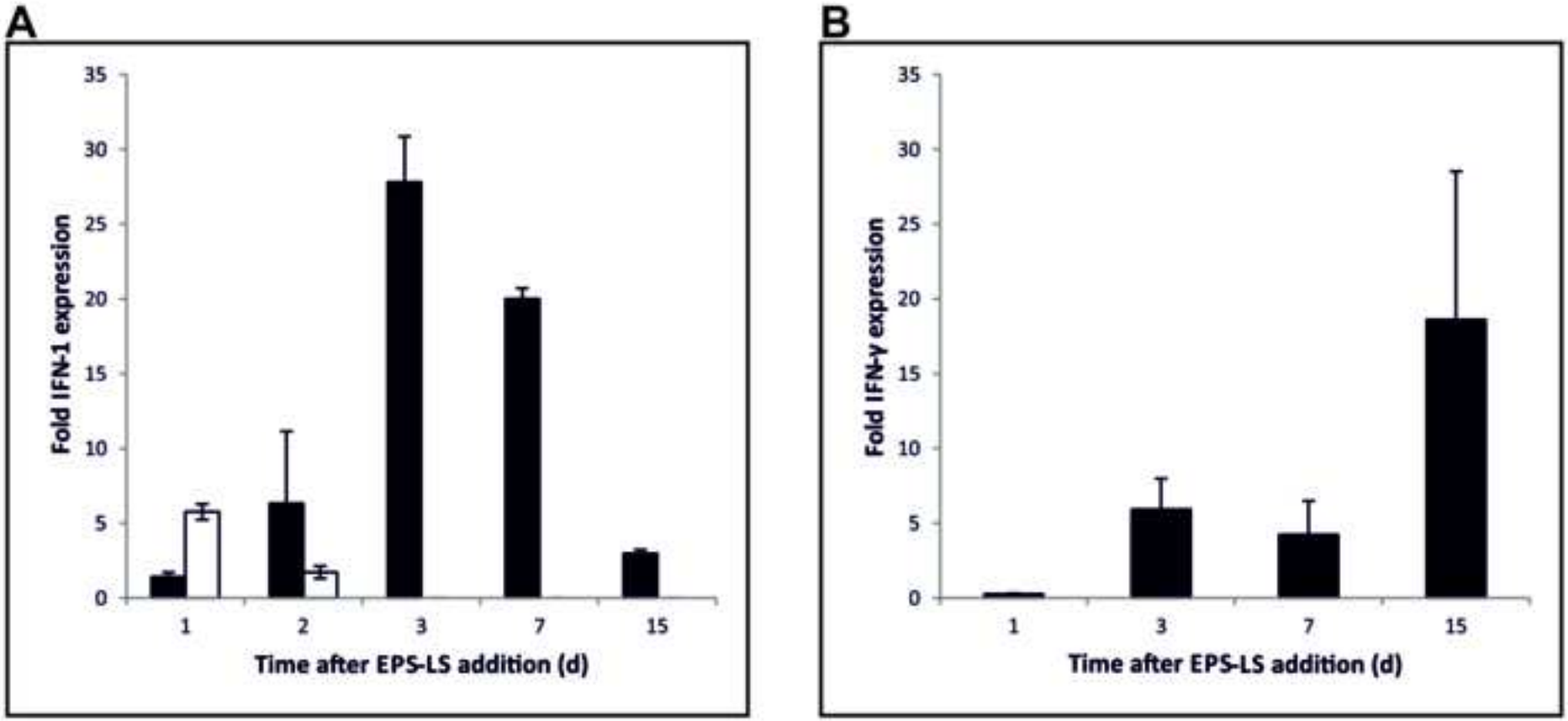

Fig. 7

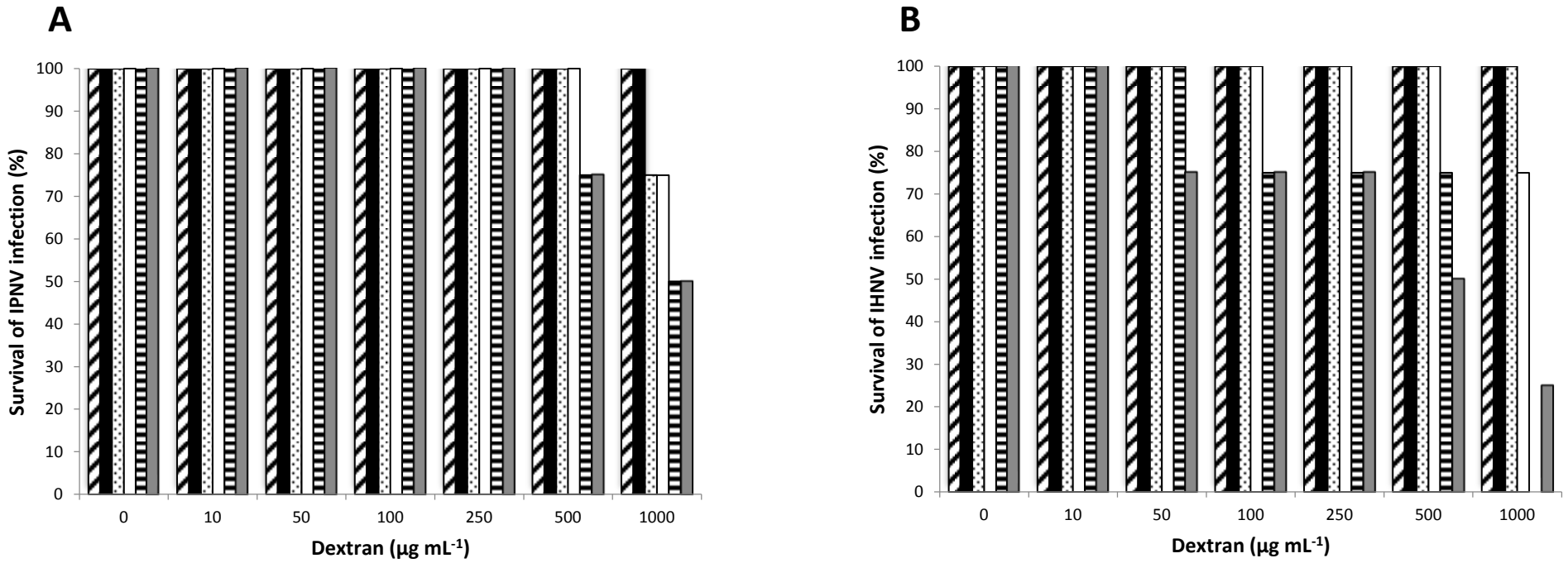

Figure 1S. Cell survival at different concentrations of dextrans against IPNV infection of BF-2 cells (A), and IHNV infection of EPC cells (B). Symbols: T10 diagonal stripes, T70 black, T500 dots, T2000 white, EPS-LM horizontal stripes and EPS-LS grey.