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Assessment of the effects of sulfated polysaccharides extracted from the red seaweed Irish moss *Chondrus crispus* on the immune-stimulant activity in mussels *Mytilus spp.*

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Abstract

Seaweeds contain a number of health enhancing and antimicrobial bioactive compounds including sulfated polysaccharides (SP). In the present study, SP extracted from a European red seaweed Irish moss *Chondrus crispus* was chemically analyzed, SP content extracted and the immune-response effect on wild Irish mussels *Mytilus* spp. investigated for the first time. A high percent yield of SP was extracted from *C. crispus* and the immune-stimulant activity of SP was assessed in a laboratory trial with mussels exposed to three different treatments of low (10 µg mL^{-1}), medium (20 µg mL^{-1}) and high (50 µg mL^{-1}) SP dose concentrations and a control mussel group with no exposure to SP. An initial mussel sample was processed prior to the trial commencing and mussels were subsequently sampled on Days 1, 2, 3, 4, 7, and 10 post SP exposure. Both cell, humoral and immune related gene responses including haemocyte cell viability, haemocyte counts, lysozyme activity and expression of immune related genes (defensin, mytimycin and lysozyme mRNA) were assessed. No mussel mortalities were observed in either the treated or non-treated groups. Mussels exposed with SP showed an increase in haemocyte cell viability and the total number of haemocytes compared to control mussels. Lysozyme activity was also higher in treated mussels. Additionally, up-regulated expression of defensin, mytimycin and lysozyme mRNA was observed in SP treated mussels shortly after exposure (on Days 1, 2, and 3) to SP. These results indicate that a high quality yield of SP can be readily extracted from *C. crispus* and more importantly based on the animal model used in this study, SP extracted from *C. crispus* can rapidly induce health enhancing activities in *Mytilus* spp. at a cellular, humoral and molecular level and with a prolonged effect up to ten days post treatment.

Keyword: *Chondrus crispus*; immune responses; *Mytilus spp*; sulfated polysaccharides

1. Introduction

Marine natural products have received much attention in recent years due to their health benefits and bioactivity. More than 16,000 natural products have been isolated from marine organisms including proteins, amino acids, lipid, fibers, pigments and polysaccharides, all of which have the potential to modulate the immune system [1,2]. In particular, seaweeds are recognized as a rich source of diverse bioactive compounds such as sulfated polysaccharides (SP). Marine seaweeds contain many different polysaccharides related to their taxonomic classifications [3]. It is acknowledged that SP from seaweed groups

include carrageenan and agaran from red seaweeds, ulvan from green seaweeds and fucoidan from brown seaweeds [4,5]. SP present in the extracellular matrix of seaweeds [6] have been increasingly reported to induce many health enhancing biological activities and are considered to be antioxidant, anticancer, anti-inflammatory, antiviral, and immunomodulatory [7]. The immunostimulatory effect induced by SP derived from marine seaweeds has been widely studied in vertebrate and other invertebrate animal models [4,8]. In other invertebrate animals, laminaran and β-glucan from the brown macroalgae *Laminaria hyperborea* showed immunomodulatory effects on Atlantic salmon *Salmo salar* macrophages [9]. Alginate extracted from seaweed increased the proportion of neutrophils, phagocytic activity, respiratory burst and expression of interleukins in rainbow trout *Oncorhynchus mykiss* post injection [10]. Administration of fucoidans from the brown macroalgae *Sargassum wightii* by immersion in Pacific white shrimp *Litopenaeus vannamei* culture was demonstrated to increase haemocyte proliferation and the mitotic index of haematopoietic tissue [11]. Oral administration of carrageenan, a red seaweed polysaccharide from *Gigartina sp.*, supplemented diets has been reported to increase the immune-related expression in *L. vannamei* [12]. Additionally, treatment of alginate and fucoidans from the brown seaweed, *Macrocystis pyrifera* and *Fucus vesiculosus* enhanced the immune response activity in head kidney leukocytes of cod, *Gadus morhua* [13].

Chondrus crispus, a red seaweed from the family *Gigartinaceae*, is found abundantly on the Atlantic coasts of Europe and North America [14]. It has been recognized that *C. crispus* is a source of SP since the 1960s [15] and besides having a relatively high SP content, *C. crispus* is also rich in proteins, amino acids, lipids and pigments [16]. The biochemical composition of SP from seaweeds is dependent on the species, anatomical regions, growing conditions, extraction procedures and analytical methods [4]. Protein content in seaweeds has not been studied as well as polysaccharide content but it is acknowledged that seaweed protein content can be species dependent with protein content in green and red seaweeds being higher (up to *35-44%* of dry mass) compared to brown seaweeds (less than *5%*) [16,17]. In addition to protein and polysaccharide content, polyphenols such as phenolic compounds (*2.5%*) and flavonoids (*0.1%*), are also produced by seaweeds [18]. In a recent study, water-soluble polysaccharides from *C. crispus* were observed to enhance the immune response in the free-living nematode *Caenorhabditis elegans* and to suppress the expression of quorum sensing and the virulence factors of the gram-negative bacteria *Pseudomonas aeruginosa* [19]. In that study, it was suggested that the water-soluble polysaccharides derived from *C. crispus* may play a health-promoting role in animals and humans. A growing

interest in the biomedical prospects of seaweed-derived SP in human health care has emphasized the need for strategies to maximize SP extraction, bioavailability and investigate precisely the therapeutic mechanisms of SP.

Mussels *Mytilus spp.* belonging to the Mytillidae family are important aquatic animals that are harvested for human consumption worldwide [20] and also play an important ecological role in the marine environment, forming biogenic reefs [21]. The blue mussel *Mytilus edulis* are boreo-temperate in their distribution on both coasts of the Atlantic Ocean in Europe and North America and are found in abundance, intertidally and subtidally, in both sheltered and exposed sites, attached to hard substrates [22,23]. The Mediterranean mussel *Mytilus galloprovincialis* is endemic to the Mediterranean, Black Sea and Adriatic Sea and has expanded its range to the British Isles [24]. Evidence of hybridisation and hybrid zones of *M. edulis* and *M. galloprovincialis* in the south west of England and Ireland were first recorded in the 1970s and subsequent studies have further documented this phenomenon [25,26,27]. The immune response of *Mytilus spp.* like other invertebrates includes a cellular response and soluble haemolymph factors including lysozyme, an antibacterial enzyme, and other antimicrobial peptides (AMPs) including defensins and mytimycins [28,29]. Haemocytes play an important role in invertebrate cellular immune response carrying out phagocytosis, melanization, encapsulation and cell-to-cell communication and indirect humoral immune response [30]. Several studies have shown that lysozymes are able to kill Gram-negative bacteria, which has been demonstrated in bivalves [31,32].

The objectives of the present study were (a) to extract, quantify and chemically analyze the SP derived from *C. crispus* and (b) assess the immune effects of exposure to the extracted SP using mussels *Mytilus spp.* as the animal model for the first time. Findings from this study will contribute to a better understanding of naturally derived biotherapeutics and their contribution not only to animal health and well-being, in particular in species involved in the aquaculture industry, but possibly to vertebrate health including humans.

2. Materials and methods

2.1. Cold water extract of sulfated polysaccharides from Chondrus crispus

Red seaweed Irish moss *C. crispus* (50 g) was collected in November 2015 from Inch Strand, Midleton Co. Cork, Ireland (51.79508, -8.180008). Seaweed samples were freshly

harvested, returned to the laboratory and sand and epiphytes were removed. The *C. crispus* was dried in an oven (35-40 °C) for 20-24 h. SP was extracted as previously described by Wongprasert et al. (2014) [33]. Briefly, the dry seaweed was ground and de-pigmented with benzene (24 h) and acetone (24 h). Five grams of de-pigmented seaweed powder was stirred at 35-40 °C in 500 mL distilled water (DW) for 4 h. The extract was diluted with 500 mL of hot water (100 °C) and centrifuged at *6000 x g* for 5 min. The seaweed residue was re-extracted again by adding 800 mL of DW and stirring for 4 h and its supernatant was filtered through a white cloth (35-48 mesh). The filtrate was allowed to cool to room temperature (RT) and was subsequently kept at -20 °C overnight. The frozen supernatant was thawed and centrifuged at *6000 x g* for 5 min to separate gel and non-gel fractions. The gel fraction was discarded and the non-gel fraction SP was collected, freeze-dried and stored at -20 °C for further study.

2.2. Chemical analysis

2.2.1. Sulfate content analysis

The sulfate content of SP was measured using a K_2SO_4 solution as a standard. Briefly, 20 mg of SP was hydrolyzed for 2 h at 100 °C in 0.5 mL of HCl (2N). The SP solution was then transferred and made to volume in a 10 mL volumetric flask. Humic substance was removed from the SP solution by centrifugation (*3000 x g*, 10 min). The 2 mL of the supernatant was diluted with 18 mL of DW and 2 mL of HCl (0.5 N). Then 1 mL of $BaCl_2$-gelatin reagent was added, swirled and retained for 30 min at RT. The mixture solution was measured at 550 nm using a spectrophotometer and the percentage of sulfate was calculated with reference to the standard curve given by a K_2SO_4 solution of known concentrations in the ranges of 10-50 μg sulfate mL^{-1}.

2.2.2. Total polysaccharide analysis

Total polysaccharide content of SP was estimated by phenol-sulfuric acid method using a galactose solution as a standard. One mL of SP solution (1 mg mL^{-1}) was mixed with 1 mL of *5%* phenol in DW and 5 mL of sulfuric acid. The mixture was vortexed and allowed to stand for 10 min at RT. The mixture solution was then cooled in an ice bath (15-20 °C) for 15 min and its absorbance was measured at 490 nm using a spectrophotometer. The

percentage of polysaccharide in SP was calculated with reference to the standard curve given by a galactose solution of known concentrations in the ranges of 10-2000 μg mL^{-1}.

2.2.3. Total protein analysis

Total protein content of SP was measured by BCA protein assay [34]. An aliquot (25 μL) of SP or standard BSA solution (final concentrations at 0-2000 μg mL^{-1}) was added to 200 μL by BCA solution (BCA™ protein assay kit, Thermo scientific, Inc., USA), mixed and incubated at 37 °C for 30 min. After incubation, the solution was measured the absorbance at 562 nm using a spectrophotometer. The total protein content of SP was calculated with reference to the standard curve given by a BSA solution of known concentrations.

2.2.4. Total phenolic analysis

The total phenolic content of SP was determined by using the Folin-Ciocalteu assay [35]. An aliquot (25 μL) of SP or standard solution of gallic acid (final concentrations at 0-100 mg L^{-1}) was added to 250 μL of DW. A reagent blank was DW. Then, 25 μL of Folin-Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 250 μL of *7 %* Na_2CO_3 solution was added to the mixture. The solution was diluted to 625 μL with DW and mixed by vortex. After incubation for 90 min at RT, the absorbance was measured at 750 nm using a spectrophotometer. Total phenolic content of SP was expressed as mg gallic acid equivalents (GAE) per 100 g of SP.

2.2.5. Total flavonoid analysis

Total flavonoid content of SP was measured by the aluminum chloride colorimetric assay [36] and modified as described by Marinova et al. (2005) [37]. An aliquot (65 μL) of SP or standard solution of catechin (final concentrations at 0-100 mg L^{-1}) was added to 325 μL of DW and mixed. Then, 19.5 μL of *5%* $NaNO_2$ was added. After 5 min, 19.5 μL of *10%* $AlCl_3$ was added. At 6 min, 130 μL of 1 M NaOH was added and the total volume was made up to 650 μL with DW. The solution was mixed and shaken and the absorbance measured at 510 nm using a spectrophotometer. Total flavonoid content of SP was expressed as mg catechin equivalents (CE) per 100 g of SP.

2.3. Immune-stimulant activity of SP derived from C. crispus in the mussel Mytilus spp.

2.3.1. Experimental design

Wild mussels *Mytilus spp.* (n=148) were randomly sampled from rocks at the intertidal at Ringaskiddy, Cork Harbour, Co. Cork (51°50'N, 8°19'W), a known hybrid zone for *M. edulis*, *M. galloprovincialis* and hybrids of both parent species, and a Class C water quality site influenced greatly by anthropogenic effects such as agricultural and industrial run-off, leachate from landfills and untreated waste water and sewage [27]. A total of 136 mussels were arbitrarily divided into 4 groups, each with duplicates of 17 mussels in stand-alone rectangular plastic aquaria containing 8 L of artificial seawater (ASW) at a salinity of 34 and at 12 ^{o}C (8 tanks in total) and aerated using an air stone. The photoperiod was 12/12 h of day/night cycles. The four groups consisted of Group 1, control mussels, not exposed to SP and Groups 2, 3 and 4 consisting of mussels exposed to SP at final concentrations of 10, 20 and 50 µg mL^{-1}, respectively. Water changes were not carried out for the trial duration to ensure that the SP dose amount added to the water at the beginning of the trial was not altered and/or removed from each system. The trial was carried out in a constant temperature room and with a constant photoperiod. Holding conditions were optimal for the mussels as no mortalities were observed in either the control or treated mussels. Mussels were fed with 2 mL of Shellfish Diet 1800 (Reed Mariculture) consisting of a heteromorphic mix of phytoplankton at a total concentration of 2 x 10^{9} cells mL^{-1} containing *Isochrysis sp*, *Pavlova sp*, *Thalossiosira weissflogii*, and *Tetraselmis sp* at day 4 of the experiment. At days 1, 2, 3, 4, 7 and 10 of the trial, 4 mussels were arbitrarily selected from each treatment (n =2/ replicate/ treatment) for immunological analyses including haemocyte viability, total haemocyte counts, lysozyme activity assay and immune related mRNA expression. All assays were performed in triplicate.

2.3.2. Cell viability in haemolymph of mussels Mytilus spp.

The neutral red retention time (NRRT) assay used to determine the haemocyte viability in mussels as previously described [38] was carried out. Briefly, haemolymph (750 µL) was collected from individual mussels using a 20-gauge sterile needle (Microlance) fitted on a 1 mL syringe containing 250 µL of tris-buffered saline solution (TBS). Haemolymph samples were constantly vortexed to resuspend haemolymph and prevent aggregation. One-

hundred microliters of haemolymph sample was then transferred into microplate well, 20 µL of *2%* neutral red solution (Sigma Aldrich, USA) was added and then sample was held in a dark humid chamber for 30 min. The supernatant was subsequently drained by tilting the plates at an angle, facilitating drainage, followed by a gentle rinse with TBS, to remove any excess neutral red dye. The neutral red was extracted by displacing the haemocytes using 100 µL of extraction solution (*1%* acetic acid in *50%* ethanol) for 30 min. The plates were measured at 450 nm and 570 nm using a UV max spectrophotometer (ELx808IU, Mason Technology, USA).

2.3.3. Total haemocyte counts

Haemolymph sample (200 µL) was individually collected and fixed with *6%* formalin in Alsever's solution (200 µL) to prevent cell aggregation. After 10 min, 20 µL portions of the fixed haemocyte suspension were mixed with 20 µL Rose Bengal solution (*1.2%* Rose Bengal in *50%* ethanol) and incubated at RT for 20 min before being used to determine the total haemocyte counts using a hemocytometer under light microscopy at 20 x magnification (Eclipse 80i microscope, Nikon Instruments Inc., USA). Haemocytometer (improved Newbauer bright line) counts were made for 5/25 squares (volume of 1 square = 0.2 x 0.2 x 0.1 mm^3) to calculate total haemocyte count mL^{-1} of hemolymph (5 x count x 10^4 x dilution factor) [39].

2.3.4. Lysozyme activity

Lysozyme activity was measured in serum as previously described by Prado-Alvarez et al. (2015) [40]. Haemolymph sample (200 µL) was individually collected from mussels and mixed with EDTA (200 µL) to prevent coagulation. Samples were centrifuged at *1200 x g* for 10 min to separate cells from the serum. Serum samples and serial dilution of standard hen egg white lysozyme suspensions (Sigma Aldrich, USA) in 0.1 M phosphate buffer (final concentrations at 0-5 µg mL^{-1}) (30 µL) were dispensed in triplicate in 96 well plates before adding a *Micrococcus luteus* (*M. lysodeikticus*) suspension (170 µL) at 0.2 mg mL^{-1} in 0.2 M monobasic sodium phosphate and 0.2 M dibasic sodium phosphate (Sigma Aldrich, USA). A set of five measurements of the optical density at 620 nm was recorded every minute using a UV max spectrophotometer (ELx808IU, Mason Technology, USA).

2.3.5. Expression of immune-related mRNA in mussels

The haemocytes from mussels (4 mussels/ group) was RNA extracted in 200 µL TRI reagents according to the manufacturer's protocol (Sigma Aldrich, USA). The concentration and quality of RNA was determined by measuring the absorbance at 260/280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA was transcribed from RNA (1 µg) using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) containing RevertAid reverse transcriptase (200 U μL^{-1}), RiboLock RNase inhibitor (20 U μL^{-1}), Oligo (dT)18 primer (100 µM), dNTP mix (10 mM), and 5X reaction buffer (250 mM tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT) at 42 °C for 1 h. The immune related mRNA was amplified by PCR using 1 µL (100 ng) of cDNA with specific primer sets and conditions (Table 1). 18S specific primer was also amplified as an internal control. Twenty-five microliters of PCR reactions contained Phusion DNA polymerase (2 U μL^{-1}), 10 mM dNTP mix, 10 µM of each forward and reverse primers and 5x Phusion HF buffer containing 7.5 mM $MgCl_2$ (Thermo Scientific, USA). The hot start PCR program used for immune related mRNA was performed with 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, annealing temperature for each particular mRNA as shown in table 1, extension at 72 °C for 30 s followed by final extension at 72 °C for 5 min. The RT-PCR product was analyzed by *1.5%* agarose gel electrophoresis, stained with *3%* ethidium bromide, and visualized under ultraviolet light and documented using the EpiChemi3 darkroom (UVP, Inc., Upland, CA). Expression was semi-quantitatively determined from the ratio of band intensity to the internal control (18S) using ImageJ analysis program (from NIH website by Scion Corporation, Frederick, MD). Each assay was carried out in triplicate.

2.4. Statistical analysis

All assays were performed in triplicate. The data were presented as mean ± SD and analyzed by one-way ANOVA followed by Tukey's multiple comparison and statistically significant difference was required at p-value less than 0.05.

3. Results

3.1. Extraction and chemical analyses of SP from C. crispus

SP extracted from the red seaweed *C. crispus* by cold water extraction was *15.36%* yield of dry weight. The chemical analysis showed that SP contained a sulfate content of *9.78 ± 0.42%* w/w, a polysaccharide content of *73.94 ± 20.61%* w/w, a total protein content of *7.08 ± 0.45%* w/w, phenolic content of *2.55 ± 0.24%* w/w and flavonoid content of *0.1002 ± 0.003%* w/w (Table 2).

3.2. C. crispus SP extract effect on immune parameters in mussel Mytilus spp.

3.2.1. Effect of SP on haemocyte cell viability in Mytilus spp. using the NRRT assay

The results of haemocyte cell viability, analysed using the NRRT assay, are shown in Fig. 1. Haemocyte cell viability of SP treated groups (final concentrations of 10, 20 and 50 µg mL^{-1}) were higher than the control group on Days 1, 2, 3, 4 and 10. While on day 7, the haemocyte cell viability was not different.

3.2.2. Effect of SP on total haemocyte counts (THCs) in Mytilus spp.

The results of THCs from SP treated groups (final concentration of 10, 20 and 50 µg mL^{-1}) and control group are shown in Fig. 2. THCs were higher in the SP exposed groups than control mussels on Days 1, 2, 3, 4 and 10, with the exception of day 7. Moreover, the results also show a positive correlation between THCs and SP dose concentration, with an increase in THCs with increasing dose concentration. For example, on Day 3, mean THCs of control and SP treated groups at the concentrations of 10, 20 and 50 µg mL^{-1} were 0.993 ± 0.24 x 10^6, 1.262 ± 0.36 x 10^6, 1.915 ± 0.15 x 10^6 and 1.724 ± 0.31 x 10^6 cells mL^{-1}, respectively. On Day 10, THCs of control and SP treated groups at the concentrations of 10, 20 and 50 µg mL^{-1} were 1.593 ± 0.47 x 10^6, 1.599 ± 0.90 x 10^6, 1.864 ± 0.32 x 10^6 and 2.263 ± 0.67 x 10^6 cells mL^{-1}, respectively.

3.2.3. Effect of SP on lysozyme activity in Mytilus spp.

Lysozyme activity of mussels treated with SP from *C. crispus* was evaluated by measuring the lysozyme activity in serum. Compared to control, lysozyme activity in the serum of mussels treated with SP significantly increased on Days 1 and 2 (Fig. 3). On Day 3, lysozyme activity in the serum of mussels treated with SP declined to control levels. On Days

4 and 7, lysozyme activity increased in treated and control groups from Day 3 but this increase was not significant in both the control and treated mussels. On Day 10, lysozyme activity in each groups had decreased from Days 4 and 7.

3.3. The effects of SP stimulation on immune related genes expression in Mytilus spp.

Post treatment with SP (final concentrations of 10, 20 and 50 µg mL^{-1}), the relative expression of defensin, mytimycin and lysozyme with 18S mRNA was evaluated in treated and control mussels on Days 1, 2, 3, 4, 7 and 10. The levels of defensin mRNA expression in mussels from the SP treated groups was significantly higher than that of mussels in the control group as shown in Fig. 4A and B. On Days 7 and 10, the levels of defensin mRNA expression showed higher than that of the control group but it was not significant. For mytimycin mRNA expression, on Days 1 and 2, the levels of mytimycin mRNA expression in mussels in the SP treated groups were significantly higher than that of mussels in the control group. On Day 3, the levels of mytimycin mRNA expression of SP treated at 50 µg mL^{-1} remained significantly high from the control group. Whereas, on Days 4, 7 and 10, the levels of mytimycin mRNA in mussels treated with the three SP concentrations showed no difference to the mussels from the control group (Fig. 4A and C). For lysozyme mRNA expression, on Days 1 and 2, the levels of lysozyme mRNA expression in mussels, from the treated groups, was significantly higher than that of mussels in the control group. On Day 3, the levels of lysozyme mRNA in mussels treated with the three SP concentrations showed no difference to the mussels from the control group. An increase of lysozyme mRNA expression in SP treated groups was observed again on Day 4. A decrease in lysozyme mRNA expression was observed from Day 7 onwards and this was relative to SP dose concentration. For example, on Day 7, the levels of lysozyme/18S mRNA expression in SP treated mussels at concentrations of 10, 20 and 50 µg mL^{-1} were 0.67, 1.1 and 2.0 folds of control, respectively and on Day 10, it was 1.6, 3.1 and 3.1 folds of control, respectively (Fig. 4A and D).

4. Discussion and Conclusion

In the present study, a high quality yield of SP from the red seaweed *C. crispus* was extracted successfully by cold-water extraction following the methodology previously reported for the red seaweed *G. fisheri* in Wongprasert et al. (2014) [33]. The yield of SP

from *C. crispus* (*15.36%*) was high and similar to other seaweeds, which have yielded a range of *14.2-19.7%*, using cold water extraction of the red seaweed *G. corticata* (*2.8-19.7%*) [41] and the green seaweed *U. pertusa* (*14.2%*) [42]. Chemical analysis in this study indicated that the SP in *C. crispus* contained high levels of carbohydrate including sulfate ester and low levels of protein, which is comparable with other red seaweeds (*Porphyra*, *Palmaria*, *Gracilaria*) [16,41]. It has been demonstrated that different thermal extractions obtain different levels of polysaccharide and sulfate contents [41], with the cold-water extraction method yielding higher levels of sulfate ester compared to the hot water extraction method, as was also observed in this study.

The immune response in *Mytilus spp.* was observed in the present study to be enhanced at a cellular, humoral and molecular level shortly after exposure to *C. crispus* SP (on day 1). Under optimal conditions, bivalves such as *Mytilus spp.* will filter seawater at a maximum rate [43] to facilitate feeding and respiration. Valve opening and continuous filtering activity was observed in the mussels in this study thus readily exposing the treated mussels to the SP dissolved in the tank seawater. Haemocyte cell viability and total haemocyte counts were increased with SP treated mussel groups compared to the nontreated mussel group. The immune system of bivalves lacks immune specificity and memory, however, innate defense mechanisms that can identify and protect against non-self-material have evolved, with invertebrate immune response centered largely on the multifunctional haemocytes [44]. Early effects of physiological alterations are often seen as changes in haemocyte counts with elevated cell counts a common response to environmental stress [45] or as observed in this study exposure to an immunostimulant biocompound. The rapid SP induced increase in haemocyte cell count and cell viability, observed in the treated mussels in this study, highlights the effective delivery mechanism of SP to the mussels via their filter feeding activity.

Similar findings including haemocyte proliferation and an increase in the mitotic index of haematopoietic tissue in response to SP exposure have been observed in crustaceans and fish species [10,11]. However, SP showed a differentially distinctive increase in percentage of total haemocyte counts, for instance *Mytilus spp*. exposed to SP from *C. crispus* and rainbow trout injected with alginate from *L. digitata* showed a similar increase in number of haemocyte. While shrimp immersion with fucodains from *S. wightii* showed increased percentage of total haemocyte counts in a lesser degree than that of mussel and fish at the same concentration and exposure time.

In the present study, lysozyme activity was measured in the serum of SP-treated compared with SP-nontreated mussels, and it was observed that lysozyme activity increased shortly (on Days 1 and 2) after exposure to SP and the level of lysozyme mRNA expression was also up-regulated on Days 1 and 2 of the trial. Previous studies reported that marine microalgae, *Chaetoceros calcitrans* (*C. calcitrans*) and *Tetraselmis suecica* (*T. suecica*), presented in Shellfish Diet 1800, have been reported to evoke an immune response in bivalves [46]. It was reported that *C. calcitrans* had a positive effect on total haemocyte count, granulocyte percentage, phagocytic rate and oxidative activity of clam haemocytes. Moreover, *T. suecica* had a positive effect on the phagocytic rate of oyster haemocytes. Interestingly, our study demonstrated that on Day 4 when the SP exposed mussels were fed the heteromorphic commercial Shellfish Diet 1800 (containing microalgae), lysozyme activity and lysozyme mRNA expression increased more once again than the nontreated control mussels, suggesting the SP possibly enhanced increased lysozyme activity after Shellfish Diet 1800 feeding.

Antimicrobial peptides such as defensins, mytilins, myticins and mytimycin, have been identified in mussel species (*M. galloprovincialis* and *Bathymodiolus azoricus*) and are associated with a humoral immune response [28]. Consistent with lysozyme mRNA, defensin and mytimycin mRNA expressions were up-regulated shortly (on Days 1, 2 and 3) after SP treatment in this study. It was reported that polysaccharides, sulfated galactans from the red seaweed *Gracilaria fisheri* stimulated immune AMPs-mRNA expressions in *L. vannamei* shrimp haemocyte [47], however the response in *Mytilus spp.* is much more rapid following exposure to SP.

In conclusion, the present study demonstrated that SP was efficiently extracted from *C. crispus* using the cold-water extraction methodology and this extracted SP has a rapid immune-stimulant effect in mussel *Mytilus spp.* This prompt immune response on a cellular, humoral and molecular level, observed in *Mytilis spp.* and instigated by SP exposure, may be beneficial to animals in assisting them in overcoming the challenges of parasites and disease. More importantly the short-term energy output required for such a rapid immune response may not be too costly to individuals whose health maybe already be compromised due to stress and poor health.

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Legends

Table 1 Specific primers and conditions used for the determination of immune-related mRNA expression in mussels *Mytilus spp.*

Table 2 Contents of sulfated polysaccharides (SP) from the red seaweed *C. crispus.* SP showed sulfate, polysaccharide, protein, phenolic and flavonoid contents. Data are presented as a mean of triplicate independent experiments.

Fig. 1. Haemocyte cell viability of mussels *Mytilus spp.* exposed with SP from the red seaweed *C. crispus* at the concentrations of 10, 20 and 50 µg mL^{-1} determined by neutral red retention time (NRPT) assay. The bar graphs show the haemocyte cell viability presented as the percent of control (mean ± standard deviation of triplicate independent experiments). * indicates value significantly different from the control ($P<0.05$), n = 4.

Fig. 2. Total haemocyte counts of mussels *Mytilus spp.* exposed with SP from the red seaweed *C. crispus* at the concentrations of 10, 20 and 50 µg mL^{-1}. Data is presented as a mean ± standard deviation of triplicate independent experiments. * indicates value significantly different from the control ($P<0.05$), n = 4.

Fig. 3. Lysozyme activity of mussels *Mytilus spp.* exposed with SP from the red seaweed *C. crispus* at the concentrations of 10, 20 and 50 µg mL^{-1}. Data is presented as a mean ± standard deviation of triplicate independent experiments. * indicates value significantly different from the control ($P<0.05$), n = 4.

Fig. 4. Expression levels of immune related mRNA of mussels *Mytilus spp.* in the initial samples, control and SP from the red seaweed *C. crispus* at the concentrations of 10, 20 and 50 µg mL^{-1}. (A) RT-PCR analysis of defensin, mytimycin and lysozyme mRNA expression by 1.5% agarose gels. (B) Densitometry value of defensin mRNA relative to 18S in different

groups, (C) densitometry value of mytimycin mRNA relative to 18S in different groups and (D) densitometry value of lysozyme mRNA relative to 18S in different groups. Bars indicate mean ± standard deviation. * indicates value significantly different from control group ($P<0.05$), n = 4.

References

[1] D.S. Bhakuni, D.S. Rawat, Bioactive marine natural products, Springer, New York. (2005) pp. 382.

[2] B. Joseph, S. Sujatha, Pharmacologically important natural products from marine sponges, J. Nat. Prod. 4 (2011) 5-12.

[3] L.G. Ferreira, M.D. Noseda, A.G. Gonçalves, D.R.B. Ducatti, M.T. Fujii, M.E.R. Duarte, Chemical structure of the complex pyruvylated and sulfated agaran from the red seaweed *Palisada flagellifera* (Ceramiales, Rhodophyta), Carbohydr. Res. 347 (1) (2012) 83-94.

[4] G. Jiao, G. Yu, J. Zhang, H. Stephen Ewart, Chemical structures and bioactivities of sulfated polysaccharides from marine algae, Mar. Drugs. 9 (2) (2011) 196-223.

[5] L. Cunha, A. Grenha, Sulfated seaweed polysaccharides as multifunctional materials in drug delivery applications, Mar. Drugs. 14 (3) (2016) 42.

[6] V.H. Pomin, P.A.S. Mourão, Structure, biology, evolution, and medical importance of sulfated fucans and galactans, Glycobiology. 18 (12) (2008) 1016-1027.

[7] C.L.F. de Almeida, H. de S. Falcão, G.R. de M. Lima, C. de A. Montenegro, N.S. Lira, P.F. de Athayde-Filho, et al., Bioactivities from marine algae of the genus *Gracilaria*, Int. J. Mol. Sci. 12 (7) (2011) 4550-4573.

[8] T. Marudhupandi, D. Inbakandan, Polysaccharides in aquatic disease management, Fish. Aquac. J. 6 (3) (2015) 135.

[9] R.A. Dalmo, R. Seljelid, The immunomodulatory effect of LPS, laminaran and sulphated laminaran [β (1, 3)-D glucan] on Atlantic salmon, *Salmo salar L.*, macrophages in vitro, J. Fish Dis. 18 (2) (1995) 175-185.

[10] S. Peddie, J. Zou, C.J. Secombes, Immunostimulation in the rainbow trout (*Oncorhynchus mykiss*) following intraperitoneal administration of Ergosan, Vet. Immunol. Immunopathol. 86 (1-2) (2002) 101-113.

[11] S. Kitikiew, J.C. Chen, D.F. Putra, Y.C. Lin, S.T. Yeh, C.H. Liou, Fucoidan effectively provokes the innate immunity of white shrimp *Litopenaeus vannamei* and its resistance against experimental *Vibrio alginolyticus* infection, Fish. Shellfish Immunol. 34 (1) (2013) 280-290.

[12] Y.Y. Chen, J.C. Chen, Y.C. Lin, D.F. Putra, S. Kitikiew, C.C. Li, et al., Shrimp that have received carrageenan via immersion and diet exhibit immunocompetence in phagocytosis despite a post-plateau in immune parameters, Fish. Shellfish Immunol. 36 (2) (2014) 352-366.

[13] A.C. Marlowe, C.C. Lazado, I. Berg, M.F. Brinchmann, V. Kiron, Influence of alginic acid and fucoidan on the immune responses of head kidney leukocytes in cod, Fish Physiol. Biochem. 37 (3) (2011) 603-612.

[14] M.D. Guiry, G.M. Guiry, *World-Wide Electronic Publication*. Galway: National University of Ireland 2016. Available online at: http://www.algaebase.org.

[15] R. Santos, P. Duarte, Marine plant harvest in Portugal, J. Appl. Phycol. 3 (1991) 11-18.

[16] S.L. Holdt, S. Kraan, Bioactive compounds in seaweed: Functional food applications and legislation, J. Appl. Phycol. 23 (3) (2011) 543-597.

[17] S.G. Leonard, T. Sweeney, K.M. Pierce, B. Bahar, B.P. Lynch, J.V. O' Doherty, The effects of supplementing the diet of the sow with seaweed extracts and fish oil on aspects of gastrointestinal health and performance of the weaned piglet, Livest. Sci. 134 (1-3) (2010) 135-138.

[18] M.J. Antonisamy, E.D.S. Raj, UV–VIS and HPLC studies on *Amphiroa anceps* (Lamarck) Decaisne, Arabian J. Chem. 9 (1) (2016) S907-913.

[19] J. Liu, J. Hafting, A.T. Critchley, A.H. Banskota, B. Prithiviraj, Components of the cultivated red seaweed *Chondrus crispus* enhance the immune response of *Caenorhabditis elegans* to *Pseudomonas aeruginosa* through the pmk-1, daf-2/daf-16, and skn-1 pathways, Appl. Environ. Microbiol. 79 (23) (2013) 7343-7350.

[20] S. Mortensen, K. Korsnes, O. Bergh, 'Eyes wide shut'-a critical view of aquaculture health management and risk factors in the 'real world', Bull. Eur. Assoc. Fish Pathol. 26 (1) (2006) 1-5.

[21] T.J. Holt, E.I. Rees, S.J. Hawkins, R. Seed, Biogenic Reefs (volume IX). An overview of dynamic and sensitivity characteristics for conservation management of marine SACs, Scottish Association for Marine Science (UK Marine SACs Project). (1998) pp. 170.

[22] W.H. Dall, A preliminary catalogue of the shell-bearing marine mollusks and Brachiopods of the Southeastern coast of the United States, with Illustrations of many of the species, *Bulletin of the United States National Museum* No. 37. Government Printing Office, Washington, DC. (1889) pp. 221.

[23] A. Sanjuan, C. Zapata, G. Alvarez, *Mytilus galloprovincialis* and *M. edulis* on the coasts of the Iberian Peninsula, Mar. Ecol. Prog. Ser. 113 (1-2) (1994) 131-146.

[24] E.M. Gosling, Systematics and geographic distribution of *Mytilus,* in: E.M. Gosling, (Ed.) The mussel *Mytilus:* ecology, physiology, genetics and culture. Amsterdam: Elsevier. (1992) pp. 1-20.

[25] E.M. Gosling, N.P. Wilkins, Phosphoglucoisomerase allele frequency data in *Mytilus edulis* from Irish coastal sites: its ecological significance, in: B.F. Keegan, P. O'Ceidigh, P.J.S. Boaden, (Ed.) Biology of Benthic Organisms: 11th European Symposium on Marine Biology, Galway. (1977) pp. 297-309.

[26] E. Gosling, S. Doherty, N. Howley, Genetic characterization of hybrid mussel (*Mytilus*) populations on Irish coasts, J. Mar. Biol. Assoc. UK. 88 (2) (2008) 341-346.

[27] S.A. Lynch, E. Morgan, J. Carlsson, C. Mackenzie, E.C. Wooton, A.F. Rowley, et al., The health status of mussels, *Mytilus spp*., in Ireland and Wales with the molecular identification of a previously undescribed haplosporidian, J. Invertebr. Pathol. 118 (2014) 59-65.

[28] G. Mitta, F. Vandenbulcke, P. Roch, Original involvement of antimicrobial peptides in mussel innate immunity, FEBS Lett. 486 (3) (2000) 185-190.

[29] L. Canesi, M. Betti, C. Ciacci, L.C. Lorusso, C. Pruzzo, G. Gallo, Cell signalling in the immune response of mussel hemocytes, Invert. Surviv. J. 3 (2006) 40-49.

[30] K. Söderhäll, Editorial. Invertebrate immunity, Develop. Comp. Immunol. 23 (4-5) (1999) 263-266.

[31] I.W. Nilsen, K. Overbo, E. Sandsdalen, E. Sandaker, K. Sletten, B. Myrnes, Protein purification and gene isolation of chlamysin, a cold-active lysozyme-like enzyme with antibacterial activity, FEBS Lett. 464 (3) (1999) 153-158.

[32] Q.G. Xue, K.L. Schey, A.K. Volety, F.L. Chu, J.F. La Peyre, Purification and characterization of lysozyme from plasma of the eastern oyster (*Crassostrea virginica*), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 139 (1) (2004) 11-25.

[33] K. Wongprasert, T. Rudtanatip, J. Praiboon, Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp, Fish. Shellfish Immunol. 36 (1) (2014) 52-60.

[34] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, et al., Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1) (1985) 76-85.

[35] V. Singleton, J. Rossi, Colorimetry of total phenolics with phosphomolibdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (3) (1965) 144-158.

[36] J. Zhishen, T. Mengcheng, W. Jianming, The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals, Food Chem. 64 (4) (1999) 555-559.

[37] D. Marinova, F. Ribarova, M. Atanassova, Total phenolics and total flavonoids in bulgarian fruits and vegetables, J. Chem. Technol. Metall. 40 (3) (2005) 255-260.

[38] M.N. Moore, J.A.J. Readman, J.W. Readman, D.M. Lowe, P.E. Frickers, A. Beesley, Lysosomal cytotoxicity of carbon nanoparticles in cells of the molluscan immune system: an in vivo study, Nanotoxicology 3 (1) (2009) 40-45.

[39] K. Sritunyalucksana, W. Gangnonngiw, S. Archakunakorn, D. Fegan, T. Flegal, Bacterial clearance rate and a new differential hemocyte staining method to assess immunostimulant activity in shrimp, Dis. Aquat. Organ. 63 (1) (2005) 89-94.

[40] M. Prado-Alvarez, S. Lynch, A. Kane, G. Darmody, B. Pardo, P. Martínez, et al., Oral immunostimulation of the oyster *Ostrea edulis*: Impacts on the parasite *Bonamia ostreae*, Fish. Shellfish Immunol. 45 (1) (2015) 43-51.

[41] S. Mazumder, P.K. Ghosal, C.A. Pujol, M.J. Carlucci, E.B. Damonte, B. Ray, Isolation, chemical investigation and antiviral activity of polysaccharide from *Gracilaria corticata* (Gracilariaceae, Rhodophyta), Int. J. Biol. Macromol. 31 (1-3) (2002) 87-95.

[42] M. Tabarsa, J.H. Han, C.Y. Kim, S.G. You, Molecular characteristics and immunomodulatory activities of water-soluble sulfated polysaccharides from *Ulva pertusa*, J. Med. Food. 15 (2) (2012) 135-144.

[43] H.U. Riisgård, P.P. Egede, I.B. Saavedra, Feeding behavior of the mussel, *Mytilus edulis*: New Observations, with a minireview of current knowledge, J. Mar. Biol. (2011) pp. 13.

606 [44] T.S. Galloway, A. Goven, Invertebrate immunotoxicity. In: Luebke, R. (Ed.),
607 Immunotoxicology and Immunopharmacology. CRC Press, Boca Raton (2006) 365-383.
608
609 [45] M. Auffret, S. Rousseau, I. Boutet, A. Tanguy, J. Baron, D. Moraga, et al., A
610 multiparametric approach for monitoring immunotoxic responses in mussels from
611 contaminated sites in Western Mediterranea. Ecotoxicol. Environ. Saf. 63 (2006) 393-405.
612
613 [46] M. Delaporte, P. Soudant, J. Moal, C. Lambert, C. Quéré, P. Miner, et al., Effect of a
614 mono-specific algal diet on immune functions in two bivalve species-*Crassostrea gigas* and
615 *Ruditapes philippinarum*, J. Exp. Biol. 206 (17) (2003) 3053-3064.
616
617 [47] T. Rudtanatip, B. Withyachumnarnkul, K. Wongprasert, Sulfated galactans from
618 *Gracilaria fisheri* bind to shrimp haemocyte membrane proteins and stimulate the expression
619 of immune genes, Fish. Shellfish Immunol. 47 (1) (2015) 231-238.
620
621 [48] H. Li, M.G. Parisi, M. Toubiana, M. Cammarata, P. Roch, Lysozyme gene expression
622 and hemocyte behaviour in the Mediterranean mussel, *Mytilus galloprovincialis*, after
623 injection of various bacteria or temperature stresses, Fish. Shellfish Immunol. 25 (1-2) (2008)
624 143-152.
625
626 [49] M.G. Parisi, A. Vizzini, M. Toubiana, G. Sarà, M. Cammarata, Identification, cloning
627 and environmental factors modulation of a αβ defensin from the Lessepsian invasive mussel
628 *Brachidontes pharaonis* (Bivalvia: Mytilidae), Invert. Surviv. J. 12 (2015) 264-273.
629
630 [50] O. Diaz de Cerio, E. Hands, J. Humble, M.P. Cajaraville, J.A. Craft, I. Cancio,
631 Construction and characterization of a forward subtracted library of blue mussels *Mytilus*
632 *edulis* for the identification of gene transcription signatures and biomarkers of styrene
633 exposure, Mar. Pollut. Bull. 71 (1-2) (2013) 230-239.

Primers	Primer sequences (5’ to 3’)	References	Annealing temp
Lysozyme-F Lysozyme-R	ATGTGGAATCTGAAGGACTTGT CCAGTATCCAATGGTGTTAGGG	[48]	60 ^{o}C for 30 s
Defensin-F Defensin-R	GTGGCGTCTGCTGGGTTT GAATGGACTTACAATGTCGATGACA	[49]	58 ^{o}C for 30 s
Mytimycin-F Mytimycin-R	CAATCCATCACTGTTGAAT ATGGTAAATCGTGTTATGAACGTG	[50]	58 ^{o}C for 30 s
18S-F 18S-R	TTACGTCGGCGCAACTTCT CTGTTCCAAGGACTTTAATG	[50]	57.6 ^{o}C for 30 s

Table 1

Contents	% of SP extract (w/w)
Yield	15.36
Sulfate	9.78 ± 0.424
Polysaccharide	73.94 ± 20.61
Protein	7.08 ± 0.455
Phenolic	2.55 ± 0.236
Flavonoid	0.10 ± 0.003

Table 2

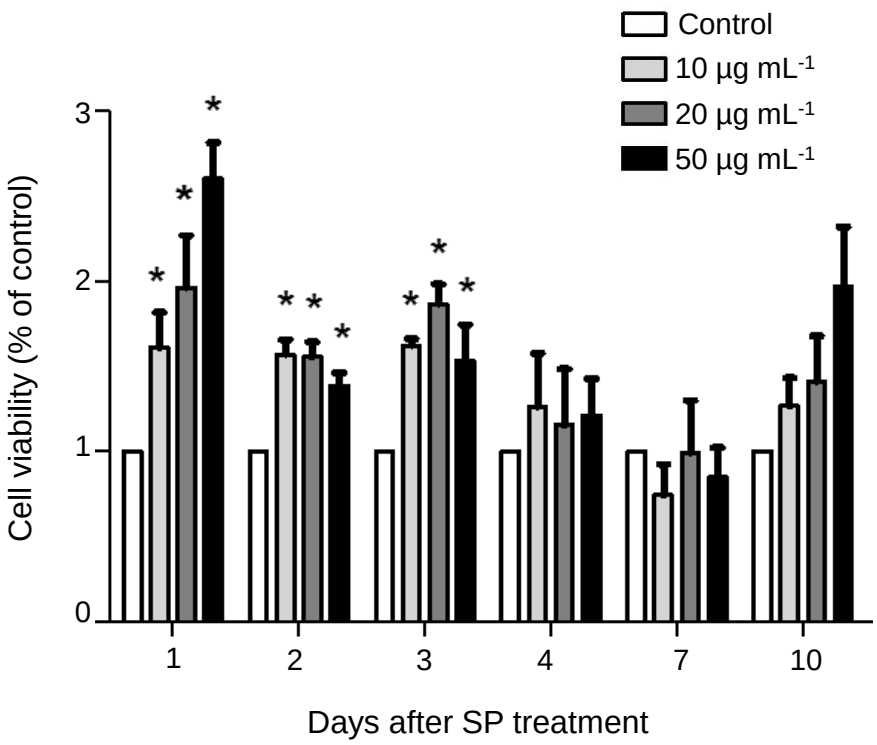
Control
10 µg mL-1
20 µg mL-1
50 µg mL-1
Cell viability (% of control)
0
1
2
3
1
2
3
4
7
10
Days after SP treatment

Figure 1

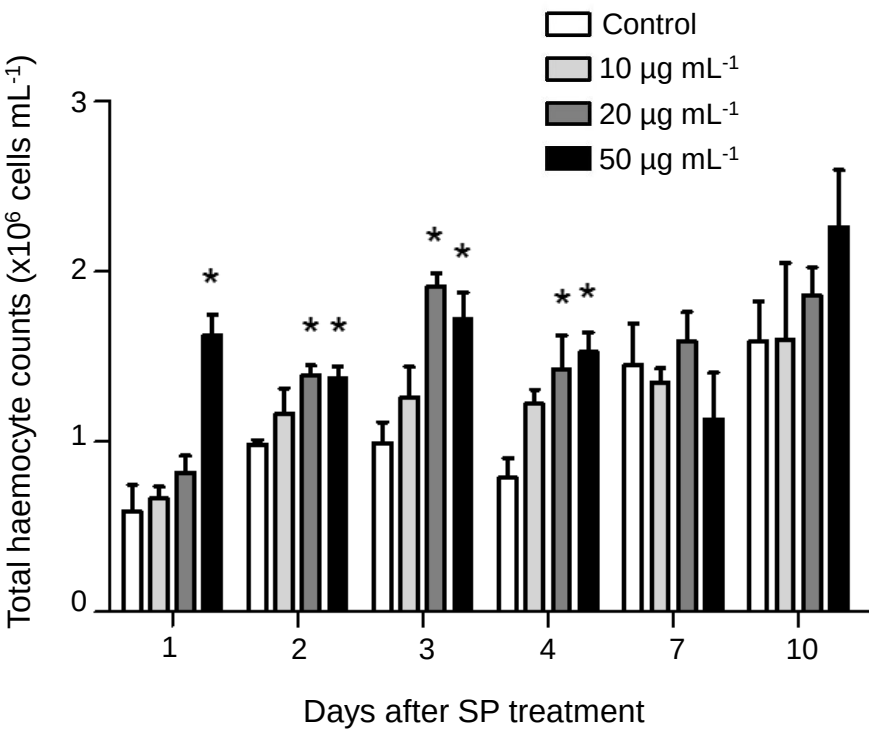
Control
10 µg mL-1
20 µg mL-1
50 µg mL-1
Total haemocyte counts (x10^6 cells mL-1)
0
1
2
3
1
2
3
4
7
10
Days after SP treatment

Figure 2

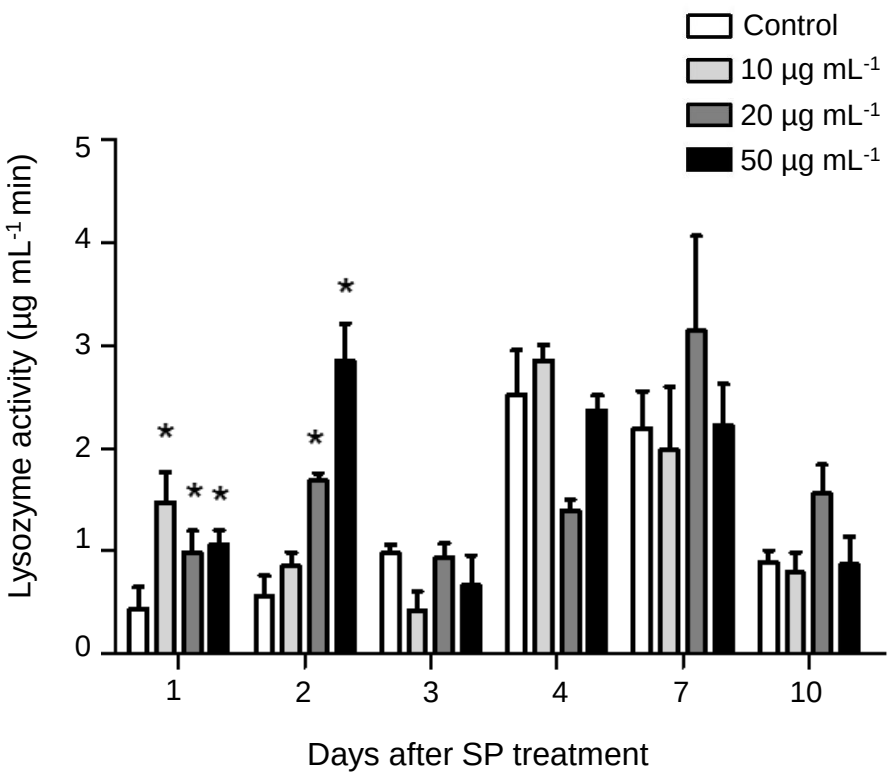
Control
10 µg mL-1
20 µg mL-1
50 µg mL-1
Lysozyme activity (µg mL-1 min)
0
1
2
3
4
5
*
*
*
*
*
1
2
3
4
7
10
Days after SP treatment

Figure 3

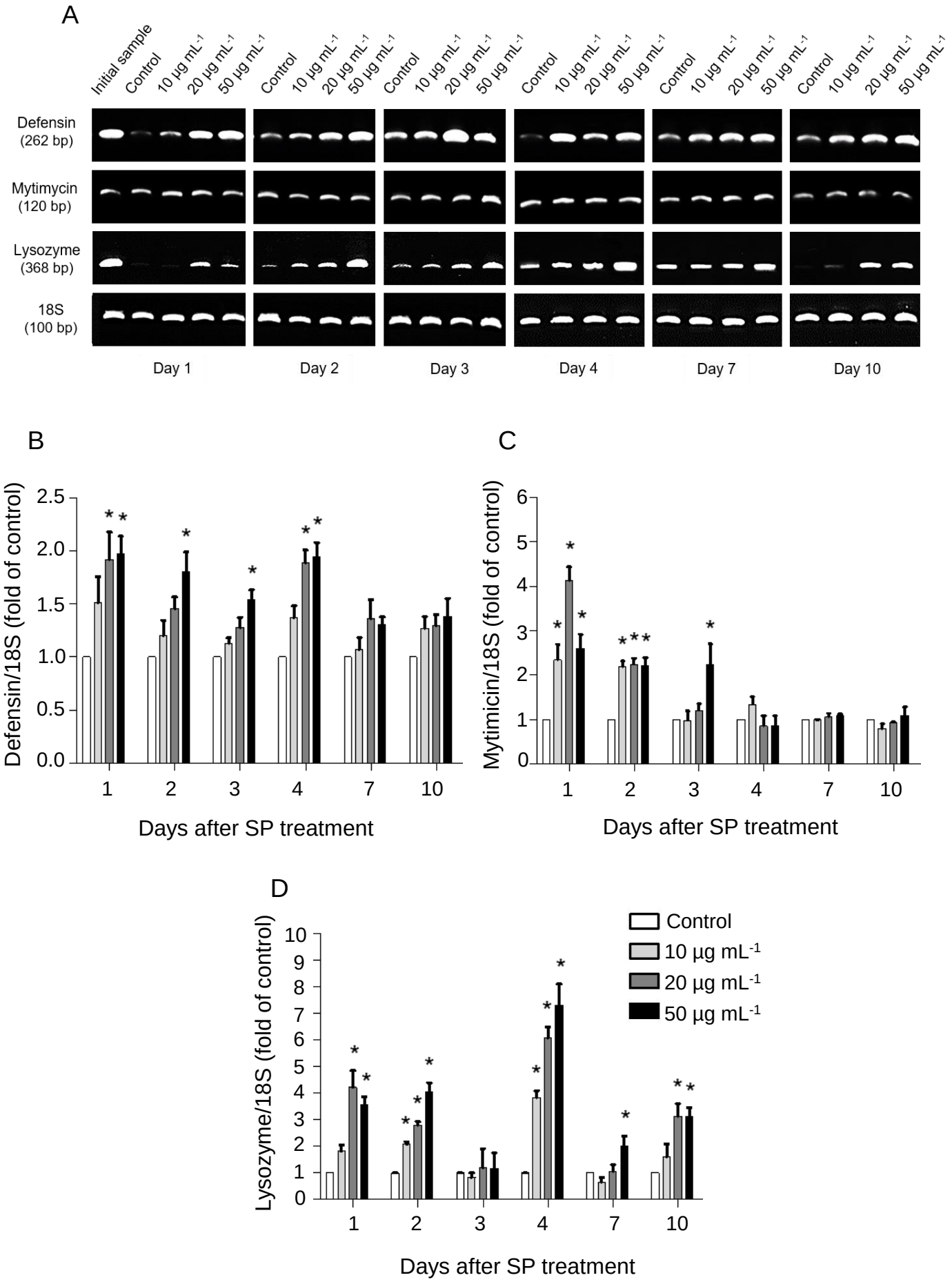
A
Initial sample
Control
10 μg mL-1
20 μg mL-1
50 μg mL-1
Defensin (262 bp)
Mytimycin (120 bp)
Lysozyme (368 bp)
18S (100 bp)
Day 1
Day 2
Day 3
Day 4
Day 7
Day 10
B
Defensin/18S (fold of control)
Days after SP treatment
C
Mytimicin/18S (fold of control)
Days after SP treatment
D
Lysozyme/18S (fold of control)
Days after SP treatment
Control
10 μg mL-1
20 μg mL-1
50 μg mL-1

Figure 4

Highlights

- Sulfated polysaccharides (SP) were extracted from Irish moss *Chondrus crispus*
- SP enhanced immune parameters in mussels *Mytilus spp.*
- SP up-regulated expression of immune genes in mussels shortly after exposure