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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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## 1 Abstract

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

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

24

## 25 1. Introduction

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

33 include carrageenan and agaran from red seaweeds, ulvan from green seaweeds and fucoidan  
34 from brown seaweeds [4,5]. SP present in the extracellular matrix of seaweeds [6] have been  
35 increasingly reported to induce many health enhancing biological activities and are  
36 considered to be antioxidant, anticancer, anti-inflammatory, antiviral, and  
37 immunomodulatory [7]. The immunostimulatory effect induced by SP derived from marine  
38 seaweeds has been widely studied in vertebrate and other invertebrate animal models [4,8]. In  
39 other invertebrate animals, laminaran and  $\beta$ -glucan from the brown macroalgae *Laminaria*  
40 *hyperborea* showed immunomodulatory effects on Atlantic salmon *Salmo salar* macrophages  
41 [9]. Alginate extracted from seaweed increased the proportion of neutrophils, phagocytic  
42 activity, respiratory burst and expression of interleukins in rainbow trout *Oncorhynchus*  
43 *mykiss* post injection [10]. Administration of fucoidans from the brown macroalgae  
44 *Sargassum wightii* by immersion in Pacific white shrimp *Litopenaeus vannamei* culture was  
45 demonstrated to increase haemocyte proliferation and the mitotic index of haematopoietic  
46 tissue [11]. Oral administration of carrageenan, a red seaweed polysaccharide from *Gigartina*  
47 *sp.*, supplemented diets has been reported to increase the immune-related expression in *L.*  
48 *vannamei* [12]. Additionally, treatment of alginate and fucoidans from the brown seaweed,  
49 *Macrocystis pyrifera* and *Fucus vesiculosus* enhanced the immune response activity in head  
50 kidney leukocytes of cod, *Gadus morhua* [13].

51 *Chondrus crispus*, a red seaweed from the family *Gigartinaceae*, is found abundantly  
52 on the Atlantic coasts of Europe and North America [14]. It has been recognized that *C.*  
53 *crispus* is a source of SP since the 1960s [15] and besides having a relatively high SP content,  
54 *C. crispus* is also rich in proteins, amino acids, lipids and pigments [16]. The biochemical  
55 composition of SP from seaweeds is dependent on the species, anatomical regions, growing  
56 conditions, extraction procedures and analytical methods [4]. Protein content in seaweeds has  
57 not been studied as well as polysaccharide content but it is acknowledged that seaweed  
58 protein content can be species dependent with protein content in green and red seaweeds  
59 being higher (up to 35-44% of dry mass) compared to brown seaweeds (less than 5%)  
60 [16,17]. In addition to protein and polysaccharide content, polyphenols such as phenolic  
61 compounds (2.5%) and flavonoids (0.1%), are also produced by seaweeds [18]. In a recent  
62 study, water-soluble polysaccharides from *C. crispus* were observed to enhance the immune  
63 response in the free-living nematode *Caenorhabditis elegans* and to suppress the expression  
64 of quorum sensing and the virulence factors of the gram-negative bacteria *Pseudomonas*  
65 *aeruginosa* [19]. In that study, it was suggested that the water-soluble polysaccharides  
66 derived from *C. crispus* may play a health-promoting role in animals and humans. A growing

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

70       Mussels *Mytilus spp.* belonging to the Mytillidae family are important aquatic animals  
71 that are harvested for human consumption worldwide [20] and also play an important  
72 ecological role in the marine environment, forming biogenic reefs [21]. The blue mussel  
73 *Mytilus edulis* are boreo-temperate in their distribution on both coasts of the Atlantic Ocean  
74 in Europe and North America and are found in abundance, intertidally and subtidally, in both  
75 sheltered and exposed sites, attached to hard substrates [22,23]. The Mediterranean mussel  
76 *Mytilus galloprovincialis* is endemic to the Mediterranean, Black Sea and Adriatic Sea and  
77 has expanded its range to the British Isles [24]. Evidence of hybridisation and hybrid zones of  
78 *M. edulis* and *M. galloprovincialis* in the south west of England and Ireland were first  
79 recorded in the 1970s and subsequent studies have further documented this phenomenon  
80 [25,26,27]. The immune response of *Mytilus spp.* like other invertebrates includes a cellular  
81 response and soluble haemolymph factors including lysozyme, an antibacterial enzyme, and  
82 other antimicrobial peptides (AMPs) including defensins and mytimycins [28,29].  
83 Haemocytes play an important role in invertebrate cellular immune response carrying out  
84 phagocytosis, melanization, encapsulation and cell-to-cell communication and indirect  
85 humoral immune response [30]. Several studies have shown that lysozymes are able to kill  
86 Gram-negative bacteria, which has been demonstrated in bivalves [31,32].

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

93

## 94 **2. Materials and methods**

95

### 96 *2.1. Cold water extract of sulfated polysaccharides from Chondrus crispus*

97

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

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

112

## 113 2.2. Chemical analysis

114

### 115 2.2.1. Sulfate content analysis

116

117 The sulfate content of SP was measured using a  $K_2SO_4$  solution as a standard. Briefly,  
118 20 mg of SP was hydrolyzed for 2 h at 100 °C in 0.5 mL of HCl (2N). The SP solution was  
119 then transferred and made to volume in a 10 mL volumetric flask. Humic substance was  
120 removed from the SP solution by centrifugation ( $3000 \times g$ , 10 min). The 2 mL of the  
121 supernatant was diluted with 18 mL of DW and 2 mL of HCl (0.5 N). Then 1 mL of  $BaCl_2$ -  
122 gelatin reagent was added, swirled and retained for 30 min at RT. The mixture solution was  
123 measured at 550 nm using a spectrophotometer and the percentage of sulfate was calculated  
124 with reference to the standard curve given by a  $K_2SO_4$  solution of known concentrations in  
125 the ranges of 10-50  $\mu g$  sulfate  $mL^{-1}$ .

126

### 127 2.2.2. Total polysaccharide analysis

128

129 Total polysaccharide content of SP was estimated by phenol-sulfuric acid method  
130 using a galactose solution as a standard. One mL of SP solution ( $1 \text{ mg mL}^{-1}$ ) was mixed with  
131 1 mL of 5% phenol in DW and 5 mL of sulfuric acid. The mixture was vortexed and allowed  
132 to stand for 10 min at RT. The mixture solution was then cooled in an ice bath (15-20 °C) for  
133 15 min and its absorbance was measured at 490 nm using a spectrophotometer. The

134 percentage of polysaccharide in SP was calculated with reference to the standard curve given  
135 by a galactose solution of known concentrations in the ranges of 10-2000  $\mu\text{g mL}^{-1}$ .

136

### 137 *2.2.3. Total protein analysis*

138

139 Total protein content of SP was measured by BCA protein assay [34]. An aliquot (25  
140  $\mu\text{L}$ ) of SP or standard BSA solution (final concentrations at 0-2000  $\mu\text{g mL}^{-1}$ ) was added to  
141 200  $\mu\text{L}$  by BCA solution (BCA<sup>TM</sup> protein assay kit, Thermo scientific, Inc., USA), mixed and  
142 incubated at 37 °C for 30 min. After incubation, the solution was measured the absorbance at  
143 562 nm using a spectrophotometer. The total protein content of SP was calculated with  
144 reference to the standard curve given by a BSA solution of known concentrations.

145

### 146 *2.2.4. Total phenolic analysis*

147

148 The total phenolic content of SP was determined by using the Folin-Ciocalteu assay [35].  
149 An aliquot (25  $\mu\text{L}$ ) of SP or standard solution of gallic acid (final concentrations at 0-100 mg  
150  $\text{L}^{-1}$ ) was added to 250  $\mu\text{L}$  of DW. A reagent blank was DW. Then, 25  $\mu\text{L}$  of Folin-Ciocalteu's  
151 phenol reagent was added to the mixture and shaken. After 5 min, 250  $\mu\text{L}$  of 7 %  $\text{Na}_2\text{CO}_3$   
152 solution was added to the mixture. The solution was diluted to 625  $\mu\text{L}$  with DW and mixed by  
153 vortex. After incubation for 90 min at RT, the absorbance was measured at 750 nm using a  
154 spectrophotometer. Total phenolic content of SP was expressed as mg gallic acid equivalents  
155 (GAE) per 100 g of SP.

156

### 157 *2.2.5. Total flavonoid analysis*

158

159 Total flavonoid content of SP was measured by the aluminum chloride colorimetric assay  
160 [36] and modified as described by Marinova et al. (2005) [37]. An aliquot (65  $\mu\text{L}$ ) of SP or  
161 standard solution of catechin (final concentrations at 0-100 mg  $\text{L}^{-1}$ ) was added to 325  $\mu\text{L}$  of  
162 DW and mixed. Then, 19.5  $\mu\text{L}$  of 5%  $\text{NaNO}_2$  was added. After 5 min, 19.5  $\mu\text{L}$  of 10%  $\text{AlCl}_3$   
163 was added. At 6 min, 130  $\mu\text{L}$  of 1 M NaOH was added and the total volume was made up to  
164 650  $\mu\text{L}$  with DW. The solution was mixed and shaken and the absorbance measured at  
165 510 nm using a spectrophotometer. Total flavonoid content of SP was expressed as mg  
166 catechin equivalents (CE) per 100 g of SP.

167



168 2.3. *Immune-stimulant activity of SP derived from C. crispus in the mussel Mytilus spp.*

169

170 2.3.1. *Experimental design*

171

172 Wild mussels *Mytilus spp.* (n=148) were randomly sampled from rocks at the  
173 intertidal at Ringaskiddy, Cork Harbour, Co. Cork (51°50'N, 8°19'W), a known hybrid zone  
174 for *M. edulis*, *M. galloprovincialis* and hybrids of both parent species, and a Class C water  
175 quality site influenced greatly by anthropogenic effects such as agricultural and industrial  
176 run-off, leachate from landfills and untreated waste water and sewage [27]. A total of 136  
177 mussels were arbitrarily divided into 4 groups, each with duplicates of 17 mussels in stand-  
178 alone rectangular plastic aquaria containing 8 L of artificial seawater (ASW) at a salinity of  
179 34 and at 12 °C (8 tanks in total) and aerated using an air stone. The photoperiod was 12/12 h  
180 of day/night cycles. The four groups consisted of Group 1, control mussels, not exposed to SP  
181 and Groups 2, 3 and 4 consisting of mussels exposed to SP at final concentrations of 10, 20  
182 and 50 µg mL<sup>-1</sup>, respectively. Water changes were not carried out for the trial duration to  
183 ensure that the SP dose amount added to the water at the beginning of the trial was not altered  
184 and/or removed from each system. The trial was carried out in a constant temperature room  
185 and with a constant photoperiod. Holding conditions were optimal for the mussels as no  
186 mortalities were observed in either the control or treated mussels. Mussels were fed with 2  
187 mL of Shellfish Diet 1800 (Reed Mariculture) consisting of a heteromorphic mix of  
188 phytoplankton at a total concentration of 2 x 10<sup>9</sup> cells mL<sup>-1</sup> containing *Isochrysis sp*, *Pavlova*  
189 *sp*, *Thalassiosira weissflogii*, and *Tetraselmis sp* at day 4 of the experiment. At days 1, 2, 3,  
190 4, 7 and 10 of the trial, 4 mussels were arbitrarily selected from each treatment (n =2/  
191 replicate/ treatment) for immunological analyses including haemocyte viability, total  
192 haemocyte counts, lysozyme activity assay and immune related mRNA expression. All  
193 assays were performed in triplicate.

194

195 2.3.2. *Cell viability in haemolymph of mussels Mytilus spp.*

196

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

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

210

### 211 2.3.3. Total haemocyte counts

212

213 Haemolymph sample (200  $\mu$ L) was individually collected and fixed with 6% formalin  
214 in Alsever's solution (200  $\mu$ L) to prevent cell aggregation. After 10 min, 20  $\mu$ L portions of  
215 the fixed haemocyte suspension were mixed with 20  $\mu$ L Rose Bengal solution (1.2% Rose  
216 Bengal in 50% ethanol) and incubated at RT for 20 min before being used to determine the  
217 total haemocyte counts using a hemocytometer under light microscopy at 20 x magnification  
218 (Eclipse 80i microscope, Nikon Instruments Inc., USA). Haemocytometer (improved  
219 Newbauer bright line) counts were made for 5/25 squares (volume of 1 square = 0.2 x 0.2 x  
220 0.1 mm<sup>3</sup>) to calculate total haemocyte count mL<sup>-1</sup> of hemolymph (5 x count x 10<sup>4</sup> x dilution  
221 factor) [39].

222

### 223 2.3.4. Lysozyme activity

224

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

235

### 2.3.5. Expression of immune-related mRNA in mussels

The haemocytes from mussels (4 mussels/ group) was RNA extracted in 200  $\mu\text{L}$  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  $\mu\text{g}$ ) using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) containing RevertAid reverse transcriptase (200 U  $\mu\text{L}^{-1}$ ), RiboLock RNase inhibitor (20 U  $\mu\text{L}^{-1}$ ), Oligo (dT)18 primer (100  $\mu\text{M}$ ), dNTP mix (10 mM), and 5X reaction buffer (250 mM tris-HCl (pH 8.3), 250 mM KCl, 20 mM  $\text{MgCl}_2$ , 50 mM DTT) at 42  $^\circ\text{C}$  for 1 h. The immune related mRNA was amplified by PCR using 1  $\mu\text{L}$  (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  $\mu\text{L}^{-1}$ ), 10 mM dNTP mix, 10  $\mu\text{M}$  of each forward and reverse primers and 5x Phusion HF buffer containing 7.5 mM  $\text{MgCl}_2$  (Thermo Scientific, USA). The hot start PCR program used for immune related mRNA was performed with 98  $^\circ\text{C}$  for 30 s, followed by 40 cycles of 98  $^\circ\text{C}$  for 10 s, annealing temperature for each particular mRNA as shown in table 1, extension at 72  $^\circ\text{C}$  for 30 s followed by final extension at 72  $^\circ\text{C}$  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  $\pm$  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*

270 SP extracted from the red seaweed *C. crispus* by cold water extraction was 15.36%  
271 yield of dry weight. The chemical analysis showed that SP contained a sulfate content of 9.78  
272  $\pm 0.42\%$  w/w, a polysaccharide content of  $73.94 \pm 20.61\%$  w/w, a total protein content of  
273  $7.08 \pm 0.45\%$  w/w, phenolic content of  $2.55 \pm 0.24\%$  w/w and flavonoid content of  $0.1002 \pm$   
274  $0.003\%$  w/w (Table 2).

275

276 3.2. *C. crispus* SP extract effect on immune parameters in mussel *Mytilus* spp.

277

278 3.2.1. Effect of SP on haemocyte cell viability in *Mytilus* spp. using the NRRT assay

279

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

284

285 3.2.2. Effect of SP on total haemocyte counts (THCs) in *Mytilus* spp.

286

287 The results of THCs from SP treated groups (final concentration of 10, 20 and 50  $\mu\text{g}$   
288  $\text{mL}^{-1}$ ) and control group are shown in Fig. 2. THCs were higher in the SP exposed groups  
289 than control mussels on Days 1, 2, 3, 4 and 10, with the exception of day 7. Moreover, the  
290 results also show a positive correlation between THCs and SP dose concentration, with an  
291 increase in THCs with increasing dose concentration. For example, on Day 3, mean THCs of  
292 control and SP treated groups at the concentrations of 10, 20 and 50  $\mu\text{g mL}^{-1}$  were  $0.993 \pm$   
293  $0.24 \times 10^6$ ,  $1.262 \pm 0.36 \times 10^6$ ,  $1.915 \pm 0.15 \times 10^6$  and  $1.724 \pm 0.31 \times 10^6$  cells  $\text{mL}^{-1}$ ,  
294 respectively. On Day 10, THCs of control and SP treated groups at the concentrations of 10,  
295 20 and 50  $\mu\text{g mL}^{-1}$  were  $1.593 \pm 0.47 \times 10^6$ ,  $1.599 \pm 0.90 \times 10^6$ ,  $1.864 \pm 0.32 \times 10^6$  and  $2.263$   
296  $\pm 0.67 \times 10^6$  cells  $\text{mL}^{-1}$ , respectively.

297

298 3.2.3. Effect of SP on lysozyme activity in *Mytilus* spp.

299

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

304 4 and 7, lysozyme activity increased in treated and control groups from Day 3 but this  
305 increase was not significant in both the control and treated mussels. On Day 10, lysozyme  
306 activity in each groups had decreased from Days 4 and 7.

307

308 *3.3. The effects of SP stimulation on immune related genes expression in Mytilus spp.*

309

310 Post treatment with SP (final concentrations of 10, 20 and 50  $\mu\text{g mL}^{-1}$ ), the relative  
311 expression of defensin, mytimycin and lysozyme with 18S mRNA was evaluated in treated  
312 and control mussels on Days 1, 2, 3, 4, 7 and 10. The levels of defensin mRNA expression in  
313 mussels from the SP treated groups was significantly higher than that of mussels in the  
314 control group as shown in Fig. 4A and B. On Days 7 and 10, the levels of defensin mRNA  
315 expression showed higher than that of the control group but it was not significant. For  
316 mytimycin mRNA expression, on Days 1 and 2, the levels of mytimycin mRNA expression  
317 in mussels in the SP treated groups were significantly higher than that of mussels in the  
318 control group. On Day 3, the levels of mytimycin mRNA expression of SP treated at 50  $\mu\text{g}$   
319  $\text{mL}^{-1}$  remained significantly high from the control group. Whereas, on Days 4, 7 and 10, the  
320 levels of mytimycin mRNA in mussels treated with the three SP concentrations showed no  
321 difference to the mussels from the control group (Fig. 4A and C). For lysozyme mRNA  
322 expression, on Days 1 and 2, the levels of lysozyme mRNA expression in mussels, from the  
323 treated groups, was significantly higher than that of mussels in the control group. On Day 3,  
324 the levels of lysozyme mRNA in mussels treated with the three SP concentrations showed no  
325 difference to the mussels from the control group. An increase of lysozyme mRNA expression  
326 in SP treated groups was observed again on Day 4. A decrease in lysozyme mRNA  
327 expression was observed from Day 7 onwards and this was relative to SP dose concentration.  
328 For example, on Day 7, the levels of lysozyme/18S mRNA expression in SP treated mussels  
329 at concentrations of 10, 20 and 50  $\mu\text{g mL}^{-1}$  were 0.67, 1.1 and 2.0 folds of control,  
330 respectively and on Day 10, it was 1.6, 3.1 and 3.1 folds of control, respectively (Fig. 4A and  
331 D).

332

#### 333 **4. Discussion and Conclusion**

334

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

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

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

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

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

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

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

401

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407 the study.

408

## 409 Legends

410

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

413

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

417

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

423

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

428

429

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

434

435 **Fig. 4.** Expression levels of immune related mRNA of mussels *Mytilus spp.* in the initial  
436 samples, control and SP from the red seaweed *C. crispus* at the concentrations of 10, 20 and  
437 50  $\mu\text{g mL}^{-1}$ . (A) RT-PCR analysis of defensin, mytimycin and lysozyme mRNA expression  
438 by 1.5% agarose gels. (B) Densitometry value of defensin mRNA relative to 18S in different



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

443

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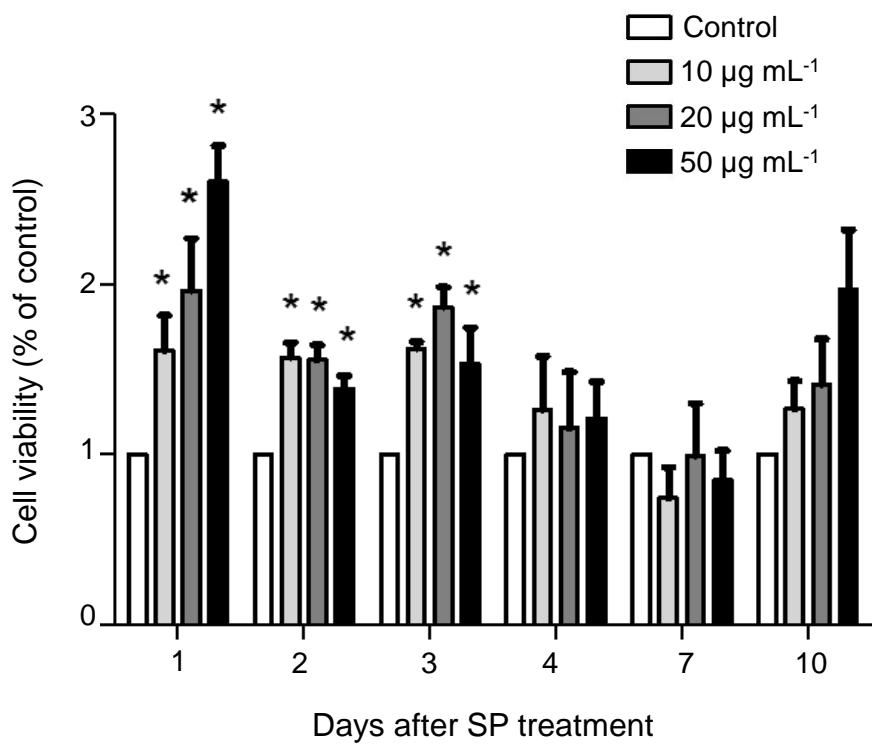
Primers	Primer sequences (5' to 3')	References	Annealing temp
Lysozyme-F Lysozyme-R	ATGTGGAATCTGAAGGACTTGT CCAGTATCCAATGGTGTTAGGG	[48]	60 °C for 30 s
Defensin-F Defensin-R	GTGGCGTCTGCTGGGTTT GAATGGACTTACAATGTCGATGACA	[49]	58 °C for 30 s
Mytimycin-F Mytimycin-R	CAATCCATCACTGTTGAAT ATGGTAAATCGTGTTATGAACGTG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

**Table 1**

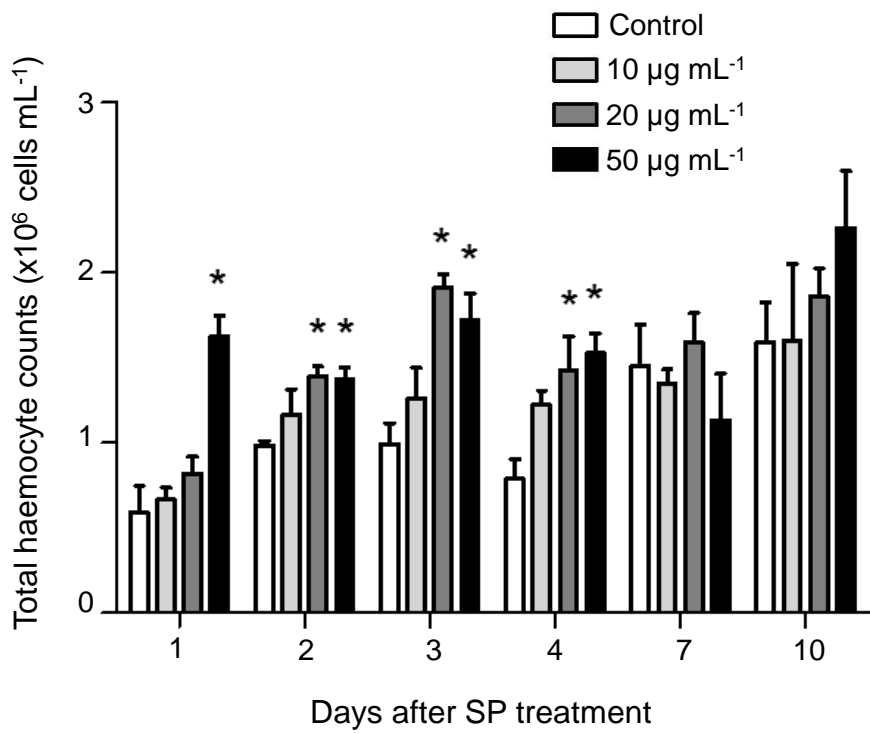
Contents	% of SP extract (w/w)
Yield	15.36
Sulfate	9.78 ± 0.424
Polysaccharide	73.94 ± 20.61
Protein	7.08 ± 0.455
Phenolic	2.55 ± 0.236
Flavonoid	0.10 ± 0.003

**Table 2**

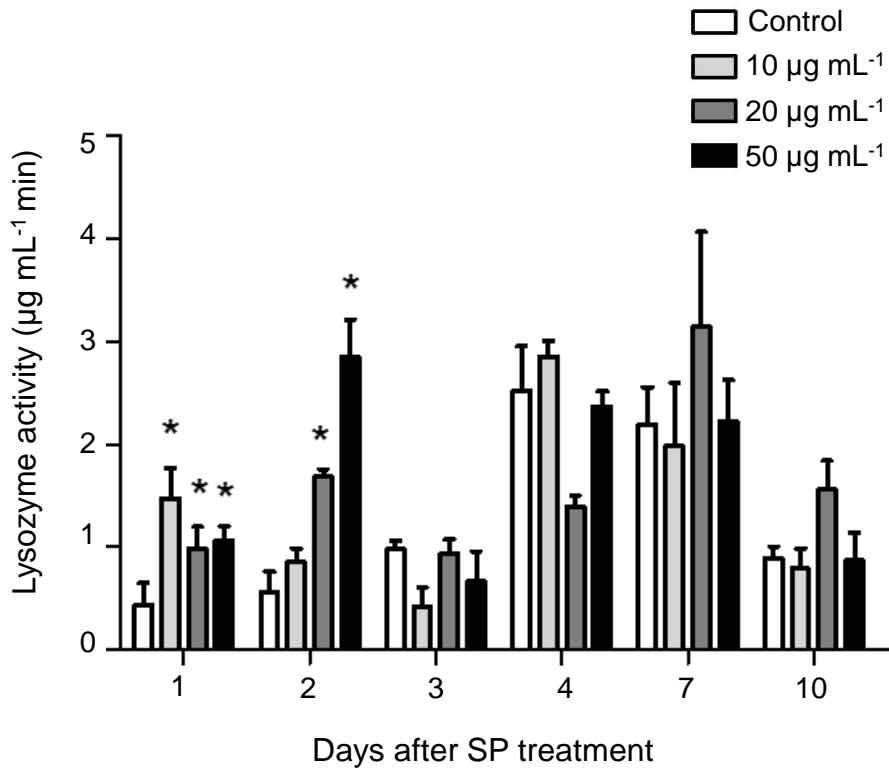




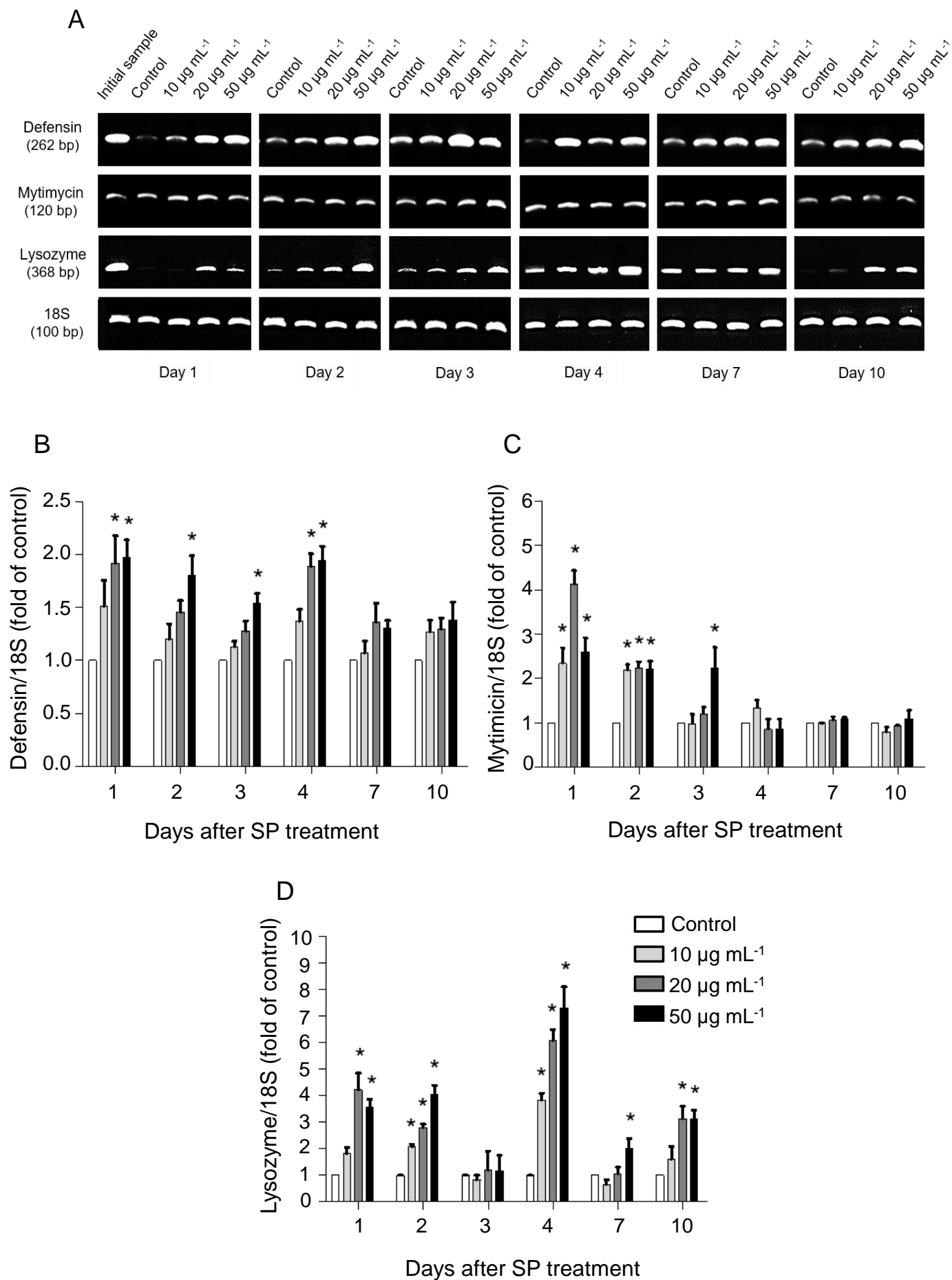
**Figure 1**



**Figure 2**



**Figure 3**



**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