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Bioencapsulation efficacy of sulfated galactans in adult *Artemia salina* for enhancing immunity in shrimp *Litopenaeus vannamei*

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ABSTRACT

Live food organisms like *Artemia* have been used for delivery of different substances such as nutrients, probiotics and immune-stimulants to aquatic animals. Previously, we reported that sulfated galactans (SG) from the red seaweed *Gracilaria fisheri* (*G. fisheri*) increased immune activity in shrimp. In the present study we further investigated the capacity and efficiency of bioencapsulation of SG in adult *Artemia* for delivery to tissues and potentially boosting the expression of immune genes in post larvae shrimp. SG were labelled with FITC (FITC-SG) for in vivo tracking in shrimp. Bioencapsulation of adult *Artemia* with FITC-SG (0–100 μg mL^{-1}) was performed and the fluorescence intensity was detected in the gut lumen after enrichment periods of 30 min, 1 h, 2 h, 6 h and 24 h. The results showed the *Artemia* took up SG over time in a concentration-dependent manner. Shrimp were fed with the bioencapsulated *Artemia* (FITC-SG, 20 μg mL^{-1}) and the shrimp were evaluated under a stereo-fluorescent microscope. At 24 h after administration, FITC-SG was located in gills and hepatopancreas and also bound with haemocytes. With daily SG administration, the genes IMD, IKKβ were up-regulated (after 1 day) while genes dicer and proPO-I were up-regulated later (after 7 days). Moreover, continued monitoring of shrimp fed for 3 consecutive days only with SG at the dose of 0.5 mg g^{-1} BW showed increases in the expression of IMD, IKKβ genes on day 1 and which gradually declined to normal levels on day 14, while the expression of dicer and proPO-I was increased on day 3 and remained high on day 14. These results demonstrate that bioencapsulation of SG in adult *Artemia* successfully delivers SG to shrimp tissues, which then bind with haemocytes and subsequently activate immune genes, and potentially increase immunity in shrimp. In addition, the present study suggests that a 3-consecutive-day regimen of SG supplemented in *Artemia* (0.5 mg g^{-1} BW) may boost and sustain the enhanced immune functions in post larvae shrimp.

1. Introduction

Immunostimulants are at the forefront of aquaculture disease management, enabling the enhancement of both humoral and cellular defense mechanisms [1]. Ringø et al. (2012) [2] described immunostimulants as an architecture consisting of repeating biodegradable units of single molecular forms such as glucose in β-glucans and riboses in DNA/RNA, fatty acid chains in bacterial lipopolysaccharides and certain lipoproteins. Immunostimulants are classed according to their composition, which includes bacterial, algae-derived, animal derived, nutritional factors and hormonal [2]. During the last two decades very intensive investigations have been carried out to define the novel category of biologically active substances that are derived from natural sources and determine varied modes and mechanism of action [3].

There are various methods of immunostimulant application within the aquaculture industry. Commercial products are readily available in liquid or powder forms based on bacterial and yeast species, and various technologies have been developed [4]. Probiotics may be administered by injection, but this method is extremely labor intensive, costly and impractical regarding shellfish culture. Furthermore, injection represents an additional stress to the cultured stock [5]. The addition of the selected candidate to the artificial diet or to culture water appears to be the preferred option within commercial shellfish farms [5]. However, delivery of the candidate by immersion or spraying usually provides better protection [6].

Bioencapsulation is the method involved in improving the nutritional and/or beneficial status of live food organisms either by feeding or integrating within them various kinds of nutrients. Live food has been used as vectors for delivering compounds of diverse nutritional value to larval stages of aquatic animals [7]. *Artemia* is widely

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recognized as the best natural storable live feed available, and is widely used in aquatic animal hatcheries around the world [8]. It can be used as a vector for the delivery of diverse materials, such as nutrients [9], antimicrobials [10,11], vaccines [12], and probiotics [13]. Previous studies have shown that *Artemia* can accumulate pharmacologic agents, such as antimicrobials [10,11]. Some of these drugs have attained therapeutic levels in fish that are fed the *Artemia* [14]. Studies with bioencapsulation of praziquantel and metronidazole in *Artemia* showed the drugs could be successfully delivered in *Artemia* with time related periods of enrichment [15,16]. Currently, research on immune stimulants in aquaculture is progressing and agents are already in use in the aquaculture industry. Sulfated galactans (SG) from the red seaweed *Gracilaria fisheri* (*G. fisheri*) were shown to exhibit immune stimulant activity by interacting with shrimp haemocyte proteins using a haemocyte culture model [17]. It has also been reported that SG enhances shrimp haemocytes, phenoloxidase activity, superoxide anions and superoxide dismutase in vivo [18]. Thus, since *Artemia* has been used successfully as a food source to deliver several natural compounds to shrimp [18–20]. The aim of the present study was to investigate whether bioencapsulated *Artemia* can deliver SG to shrimp tissues in a way that enhances and sustains shrimp immunity.

2. Materials and methods

2.1. Sulfated galactans (SG) and conjugation of SG with fluorescein isothiocyanate (FITC-SG)

Sulfated galactans (SG) was extracted from red seaweed *G. fisheri* and purified following the method previously described [17,18]. SG conjugated FITC was prepared following the method of Tanaka et al. (2004) [21]. One gram of SG was dissolved in methyl sulphoxide (10 mL) containing 3 drops of pyridine. FITC (400 mg) and dibutylin dilaurate (20 mg) were added and the mixture was heated for 2 h at 95 °C. After several precipitations in absolute ethanol, the FITC-SG was isolated, purified by size-exclusion chromatography on Sephadex G-25, freeze-dried and stored at −20 °C in the dark until used.

2.2. FITC-SG bioencapsulation in Artemia

Adult *Artemia salina* were hatched from brine shrimp cysts (Brine Shrimp Eggs, Aqua Brand, USA) in artificial sea water (ASW). Fifty grams of brine shrimp cysts were decapsulated with 50% sodium hypochlorite for 10 min, washed with tap water and transferred into a tank containing 800 mL ASW, salinity at 35 ppt. The water was aerated with mild air flow, to give enough air to the organism, and at the temperature of 28 °C for hatching. After 24 h of incubation, the nauplii were developed and then separated to a new tank containing 800 mL ASW and further cultured to develop adult *Artemia* for 10–15 days.

Artemia were enriched with FITC-SG as described previously [19]. Bioencapsulation of SG in *Artemia* was optimized with respect to time and concentration. Briefly, 100 mg of *Artemia* were enriched with FITC-SG (1–100 μg mL^{-1}) by immersion in 300 mL ASW for different periods of time (0–24 h) in the dark. The bioencapsulated *Artemia* were collected, washed carefully, and uptake of FITC-SG into the gut lumen was observed using a stereo-fluorescent microscope (Olympus, Tokyo, Japan). SG concentrations were assessed by measuring the FITC intensity using a spectrofluorometer (JASCO FP-6200 Model, Tokyo, Japan). The amount of SG in *Artemia*, represented by the fluorescence intensity, was averaged from 10 individual *Artemia*. The bioencapsulated SG content was confirmed by correlating values with the carbohydrate content in the *Artemia*, which was determined by the phenol-sulfuric acid method using galactose as a standard [22].

2.3. Tracking of SG in the shrimp

The specific pathogen-free post larvae shrimp *Litopenaeus vannamei* (*L. vannamei*), averaged weight of 150–200 mg, were obtained from Charoen Pokphand Foods (CPF), Mae Klong district, Samut Songkhram province, Thailand. Shrimp were kept in bio-filter laboratory tanks containing ASW at 28 °C, fed with normal adult *Artemia*, and acclimated for 7 days before experiments started.

In order to track the path of SG in shrimp tissues, *Artemia* were bioencapsulated with either SG or FITC-SG at 20 μg mL^{-1} for 6 h before administration to shrimp. A total of 270 shrimp were randomly divided into 3 treatment groups, each group with triplicates of 30 shrimp in stand-alone rectangular plastic tank containing 11 L ASW (9 tanks in total). Group 1 comprised shrimp which were administered with normal *Artemia* as a normal control. Group 2 comprised shrimp which were administered with *Artemia* bioencapsulated with SG as a negative FITC control. Group 3 comprised shrimp which were administered with *Artemia* bioencapsulated with FITC-SG. Shrimp (n = 5) were collected before the experiment started and shrimp from each treatment (n = 5/replicate/treatment) were collected at 15, 30 min, 1, 2 and 24 h after *Artemia* administration and fluorescence in the shrimp tissues was observed under a stereo-fluorescent microscope (Olympus, Tokyo, Japan). In order to investigate the binding of FITC-SG with haemocytes, haemolymph was collected from each group (n = 5) at 24 h. Haemolymph (20 μL) was withdrawn from the ventral sinus of shrimp into a 1 mL syringe containing 20 μL shrimp salt solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM HEPES), mixed immediately by inverting the syringe, incubated for 10 min on ice in the dark and observed using a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

2.4. Expression of immune related genes after SG administration

The immunostimulant activity of SG was evaluated in shrimp fed with *Artemia* bioencapsulated with SG by determining the level of expression of immune related genes. Our previous study revealed that shrimp fed with *Artemia* enriched with SG at 200 μg mL^{-1} (final concentration) showed a significant increase in immune activities compared to control shrimp [18]. The calculated 200 μg mL^{-1} of SG, weight for weight, was equal to 0.50 mg g^{-1} BW, and was used in the following assays. A total of 120 shrimp were divided into control and SG groups (each group with triplicates of 20 shrimp in stand-alone rectangular plastic tank containing 11 L ASW, 6 tanks in total). The shrimp were daily fed with normal *Artemia* or *Artemia* bioencapsulated with SG for 1 or 3 or 7 days after which the SG feeding was stopped. Shrimp from each treatment (n = 5/replicate/treatment) were then collected, and RNA was extracted in order to determine the expression of immune related genes, IMD, IKKβ, dicer and proPO-I by using the relative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

RNA was extracted from the shrimp in 200 μL TRI reagent according to the manufacturer's protocol (Molecular research center, Inc., 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). The RNA samples had an OD_{260}:OD_{280} between 1.8 and 2.0, indicating clean RNA. The RNA quality was also checked by 1.0% agarose gel electrophoresis, stained with 1 μg mL^{-1} ethidium bromide. RNA (5 μL, 200 ng μL^{-1}) was reverse-transcribed to cDNA using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) containing 1 μL of RevertAid reverse transcriptase (200 U μL^{-1}), 1 μL of RiboLock RNase inhibitor (20 U μL^{-1}), 1 μL of Oligo (dT)18 primer (100 μM), 2 μL of dNTP mix (10 mM), 6 μL of nuclease free water, and 4 μL of 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, followed by 70 °C for 5 min. The immune related genes were amplified by PCR using 1 μL of cDNA with the specific primer sets and conditions (Table 1). The shrimp EF-1α gene was amplified as an internal control. A 25 μL PCR reaction contained 0.125 μL of Phusion DNA polymerase (2 U μL^{-1}), 0.25 μL of 10 mM dNTP mix, 0.625 μL of 10 μM forward and reverse primers, 7.375 μL of nuclease free water, and 2.5 μL of 5X Phusion HF buffer containing 7.5 mM

Table 1
Specific primers and conditions used for determination of the expressions of signaling mediators and immune related genes.

Gene names	Primers	Nucleotide sequences (5' to 3')	Annealing temp/cycles
IMD	Forward	TGGGTCCGTGTCCAGTGAT	57 °C for 30 s
	Reverse	ACAAACAACCACACACAAGCAG	
IKKβ	Forward	ACCACACTTTCCACCTTTGG	35 cycles
	Reverse	TCCCGATGAAGGAAGAACAC	
Dicer	Forward	CCGGAGATAGAACGGTTCAGTG	60 °C for 1min
	Reverse	CGATAATTCCTCCCAACACCTG	40 cycles
proPO-I	Forward	GCCTTGGCAACGCTTTCA	58 °C for 30 s
	Reverse	CGCGCATCAGTTCAGTTTGT	40 cycles
EF-1α	Forward	GAAGTAGCCGCCCTGGTTG	57 °C for 30 s
	Reverse	CGGTTAGCCTTGGGGTTGAG	28 cycles

$MgCl_2$ (Thermo Scientific, www.thermoscientific.com/onebio). The PCR product (5 μL) was electrophoresed on 1.5% agarose gel, stained with ethidium bromide, visualized under ultraviolet light and documented using the EpiChemi3 darkroom (UVP, Inc., Upland, CA). Gel images were digitally captured and analyzed with Scion Image Software Package. Densitometry of an immune gene was normalized against the internal control gene, EF-1α and the RT-PCR value is presented as fold of control group.

2.5. Determination of the period of enhanced immune activity by SG

A total of 180 shrimp were divided into control and SG groups (each group with triplicates of 30 shrimp in stand-alone rectangular plastic tank, 6 tanks in total). The shrimp were consecutively administered with normal *Artemia* or *Artemia* bioencapsulated with SG for 3 days and then shrimp from each treatment (n = 5/replicate/treatment) were collected and expression of the immune related genes was determined at day 1, 3, 7 and 14 post 3-day-SG administration (Fig. 1B).

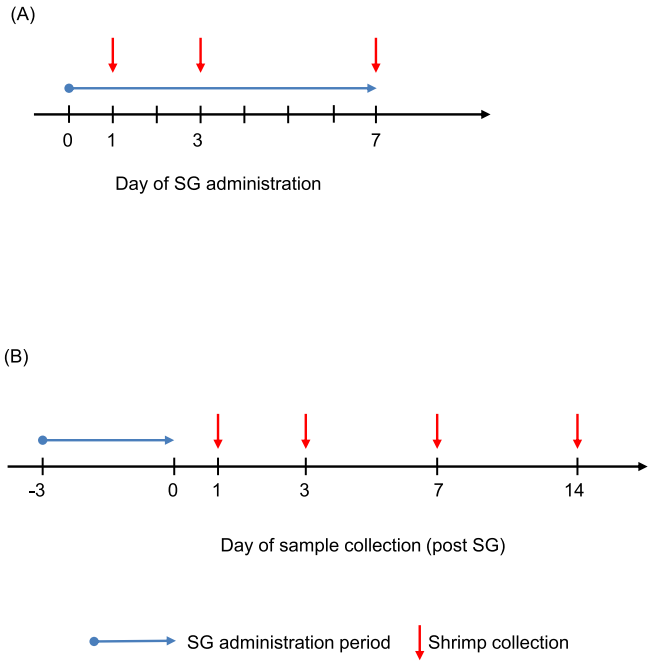

Fig. 1. Diagram showing the time-frame of experiments to determine immune expression in shrimp with different SG administration protocols. (A) Expression of immune related genes after daily SG administration for 1, 3 and 7 days. (B) Expression of immune related genes after a 3-consecutive day of SG administration to shrimp. Shrimp were collected at day 1, 3, 7 and 14 post SG.

2.6. Statistical analysis

All experiments 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. Artemia uptaking FITC-SG in a dose- and time-related fashion

Toxicity of FITC-SG in *Artemia* was investigated by immersing the *Artemia* in the FITC-SG solution at concentrations ranging from 0 to 100 μg mL^{-1} for 24 h. The results revealed that FITC-SG exposed *Artemia* showed viabilities similar to control.

Artemia were immersed in the FITC-SG solution at a concentration of 20 μg mL^{-1} and then the optimal bioencapsulation period was evaluated. At 30 min of enrichment, FITC-SG presented in the gut lumen of the *Artemia* and uptake was gradually increased with time. After 6 h of enrichment, the fluorescein was fully accumulated in the gut lumen of the *Artemia* and maintained up to 24 h of enrichment (Fig. 2A). In addition, the carbohydrate content or SG in *Artemia* was found to increase concurrently with the FITC intensity (Fig. 2B). This result suggested that the minimum gut loading period of SG was 6 h. Immersion of the *Artemia* with FITC-SG for 6 h revealed, by naked eye, the full fluorescent loading in the gut lumen at concentrations of 20–100 μg mL^{-1} (Fig. 3A). However, the FITC-SG fluorescent intensity in *Artemia* increased in a dose-dependent manner from 1 to 40 μg mL^{-1}. The enrichment concentrations from 40 to 100 μg mL^{-1} produced similar fluorescent intensities suggesting that *Artemia* enrichment was complete at 40 μg mL^{-1} (Fig. 3B). Concurrently, carbohydrate content in *Artemia* enriched with SG for 6 h increased in accordance with the fluorescent intensity of SG. These data showed that the optimum enrichment period of SG at a concentration of 40 μg mL^{-1} was 6 h. Enrichment with lower concentrations of SG, such as 20 μg mL^{-1}, would require a longer enrichment period.

3.2. FITC-SG passed from GI to tissues and bound with shrimp haemocytes

Artemia bioencapsulated with FITC-SG (20 μg mL^{-1}) was administered to the shrimp. One shrimp was administered with approximately 600 *Artemia* (3 g of *Artemia* per shrimp) calculated to equal 0.5 mg g^{-1} BW, using the results of FITC-SG intensity and carbohydrate content in *Artemia* after enrichment with SG at a concentration of 20 μg mL^{-1} for 6 h (Table 2). FITC-SG in the shrimp was observed under a stereo-fluorescent microscope at 15, 30 min, 1, 2 and 24 h after administration. The FITC-SG fluorescence was clearly visualized in the intestinal tract of the shrimp after 15 min and maintained until 2 h then disappeared (Fig. 4). The migration of FITC-SG in the shrimp to haemolymph (haemocyte), gills and hepatopancreas was investigated at 24 h after administration. The result revealed that the FITC-SG fluorescence was present in the haemolymph and bound with the haemocytes (Fig. 5). This suggested that SG passed from the GI tract of the shrimp, entered the circulation and bound with the haemocytes. In addition, FITC-SG fluorescence also presented in the gills and hepatopancreas (Fig. 6).

3.3. SG bioencapsulated in Artemia differentially upregulated expressions of immune related genes in shrimp

A daily feeding trial with SG (0.50 mg g^{-1} BW) was carried out to determine the time required to enhance the expression of immune related genes in shrimp. The results showed that the IKKβ gene was upregulated to about 3 fold of control on day 1 and this level of expression was sustained through day 7. The IMD gene was significantly upregulated early on day 1, about 1.8 fold of control shrimp, and its

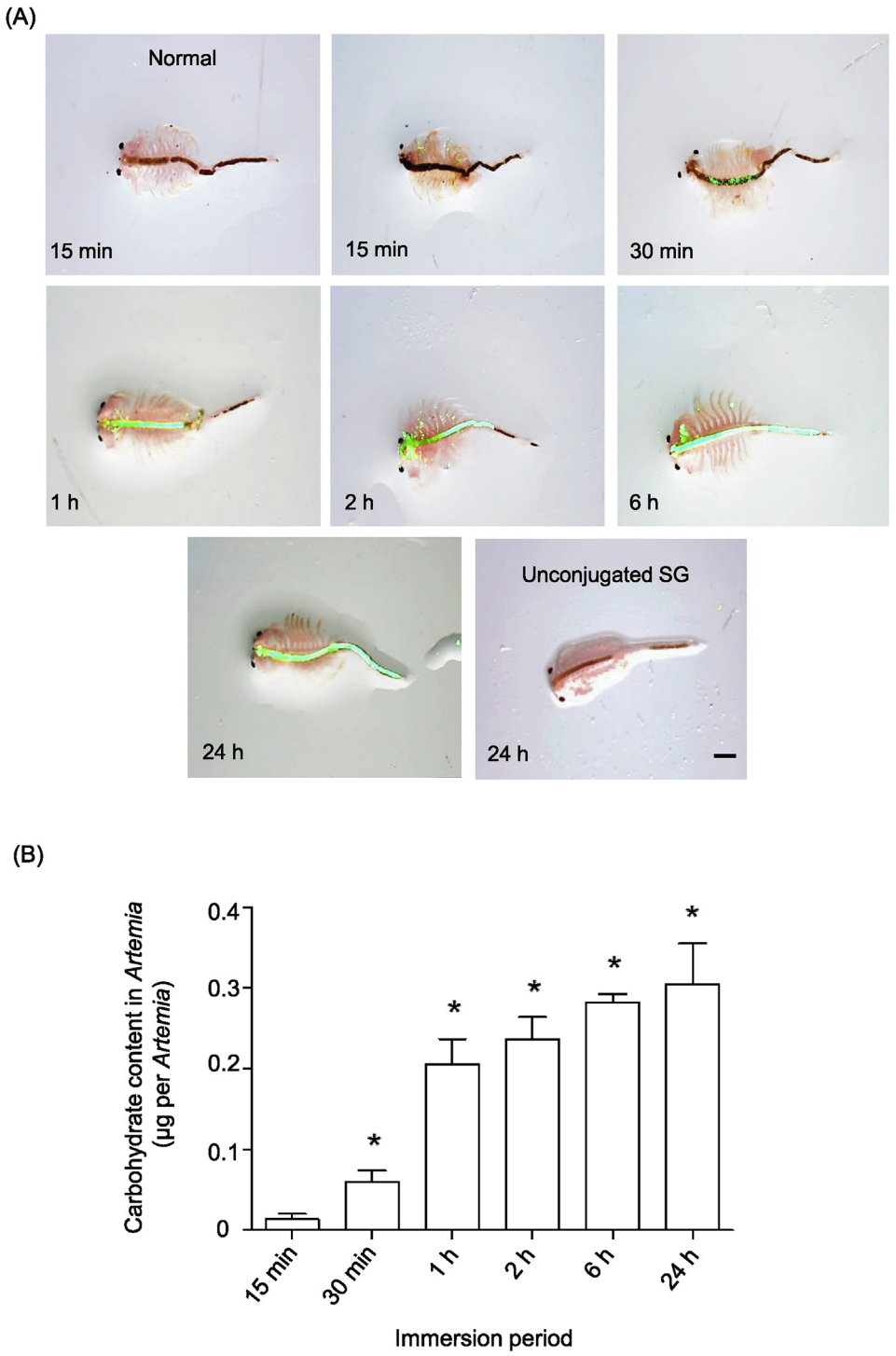

Fig. 2. FITC-SG (20 μg mL^{-1}) loading in *Artemia* at different time points observed under a stereo-fluorescent microscope. (A) FITC-SG loading in the gut lumen of *Artemia* at different time points. Scale bar = 1 mm. (B) Carbohydrate content of SG (20 μg mL^{-1}) in *Artemia* measured by phenol-sulfuric acid method. Data are presented as a mean of three independent experiments. Bars indicate mean ± standard deviation. * indicates value significantly different from 15 min ($P < 0.05$), n = 10.

expression continued to increase to about 2.6 fold of control on day 7. The expression of dicer and proPO-I genes was up-regulated later on day 3, and there was a significant increase of about 1.5 fold of control by day 7 (Fig. 7A). Importantly, a sustainable period of enhanced immunity was noted following the 3-consecutive-day feeding. Shrimp were daily administered with *Artemia* bioencapsulated with SG for 3-consecutive days and then further fed with *Artemia* without SG for an additional 1, 3, 7 and 14 days (post SG). The results demonstrated that the expression of IKKβ and IMD was significantly up-regulated on day 1 post SG and subsequently declined to the control level on day 3 and 7, respectively. Although dicer and proPO-I were expressed at a level consistent with the control on day 1 post SG, their expression was up-regulated on day 3 post SG and was sustained through day 14 (Fig. 7B). These results indicated that SG stimulates a differential immune response in the shrimp; the IKKβ and IMD were the early responsive genes and their levels declined quickly while dicer and proPO-I were the late

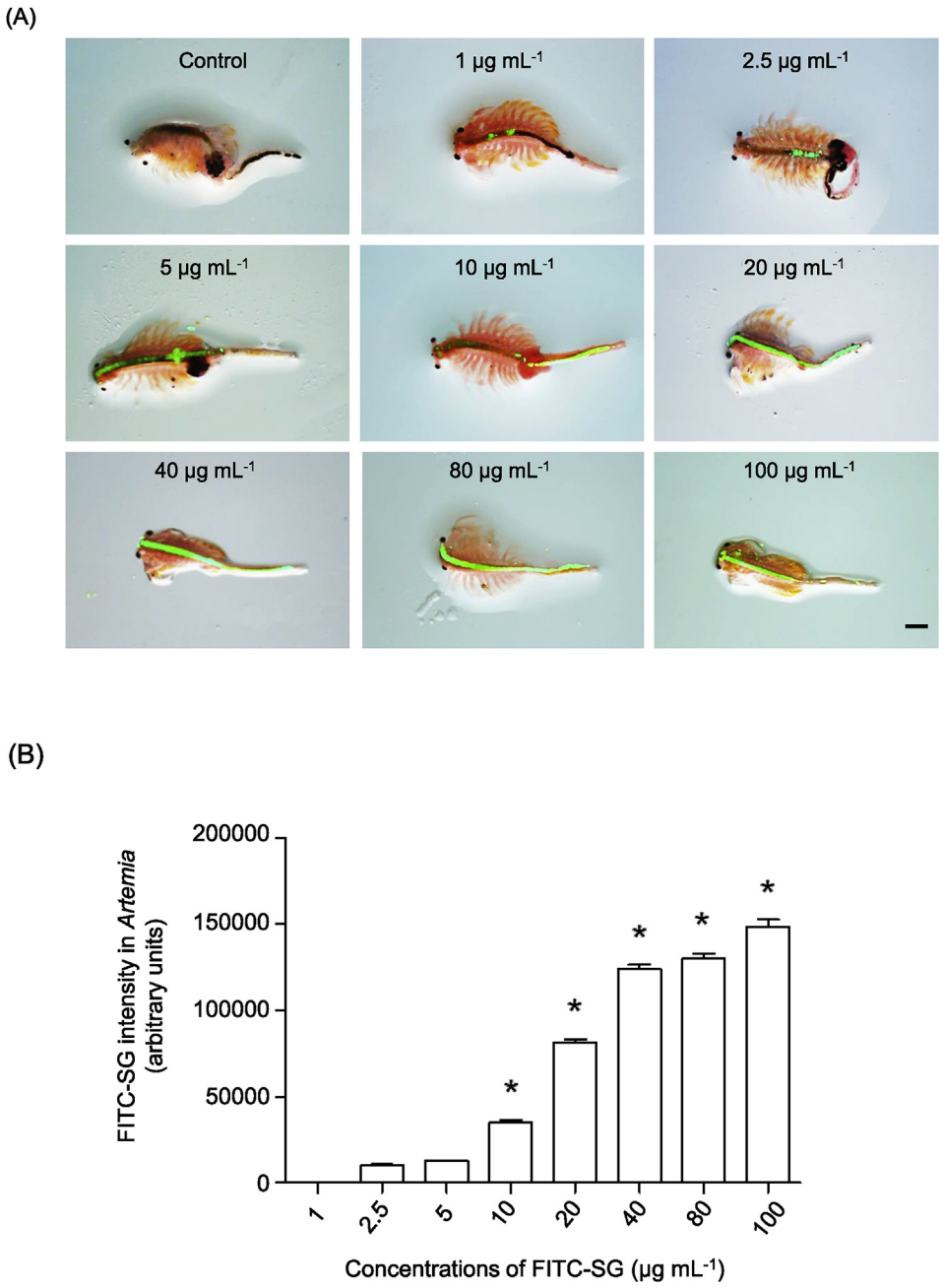

Fig. 3. Different concentrations (0–100 μg mL^{-1}) of FITC-SG in *Artemia* at 6 h of enrichment. (A) Fluorescein FITC-SG in the gut lumen of *Artemia* observed under a stereo-fluorescent microscope. Scale bar = 1 mm. (B) Fluorescent intensity of FITC-SG in *Artemia* detected by spectrofluorometer. Data are presented as a mean of three independent experiments. Bars indicate mean ± standard deviation. * indicates value significantly different from 2.5 μg mL^{-1} group ($P < 0.05$), n = 10.

Table 2
FITC-SG intensity and carbohydrate content in *Artemia* after enrichment with the different concentrations (0–100 μg mL^{-1}) of FITC-SG and SG for 6 h, determinations by spectrofluorometer and phenol-sulfuric acid methods, respectively.

Concentration of SG (μg mL^{-1})	FITC-SG content (μg per *Artemia*)	Carbohydrate content (μg per *Artemia*)
10	0.151 ± 0.01	0.109 ± 0.08
20	0.353 ± 0.02	0.205 ± 0.13
40	0.539 ± 0.02	0.306 ± 0.10
80	0.567 ± 0.02	0.321 ± 0.07
100	0.643 ± 0.04	0.355 ± 0.06

responsive genes and their levels were sustained through day 14.

4. Discussion and conclusion

Delivery of immunostimulants via oral ingestion has been accepted as the most economically viable method for extensive aquaculture systems [23]. *Artemia* is widely recognized as the best natural storable live feed available, and is widely used in aquatic animal hatcheries [8]. It can be used as a vector for the delivery of diverse materials, such as nutrients, antimicrobials, vaccines and probiotics [11,24]. Some of these compounds in *Artemia* have attained therapeutic levels in fish [14], and in shrimp [18–20]. Previously, we reported that sulfated galactans (SG) from the red seaweed *Gracilaria fisheri* (*G. fisheri*) increased immune activity in shrimp together with haemocyte counts,

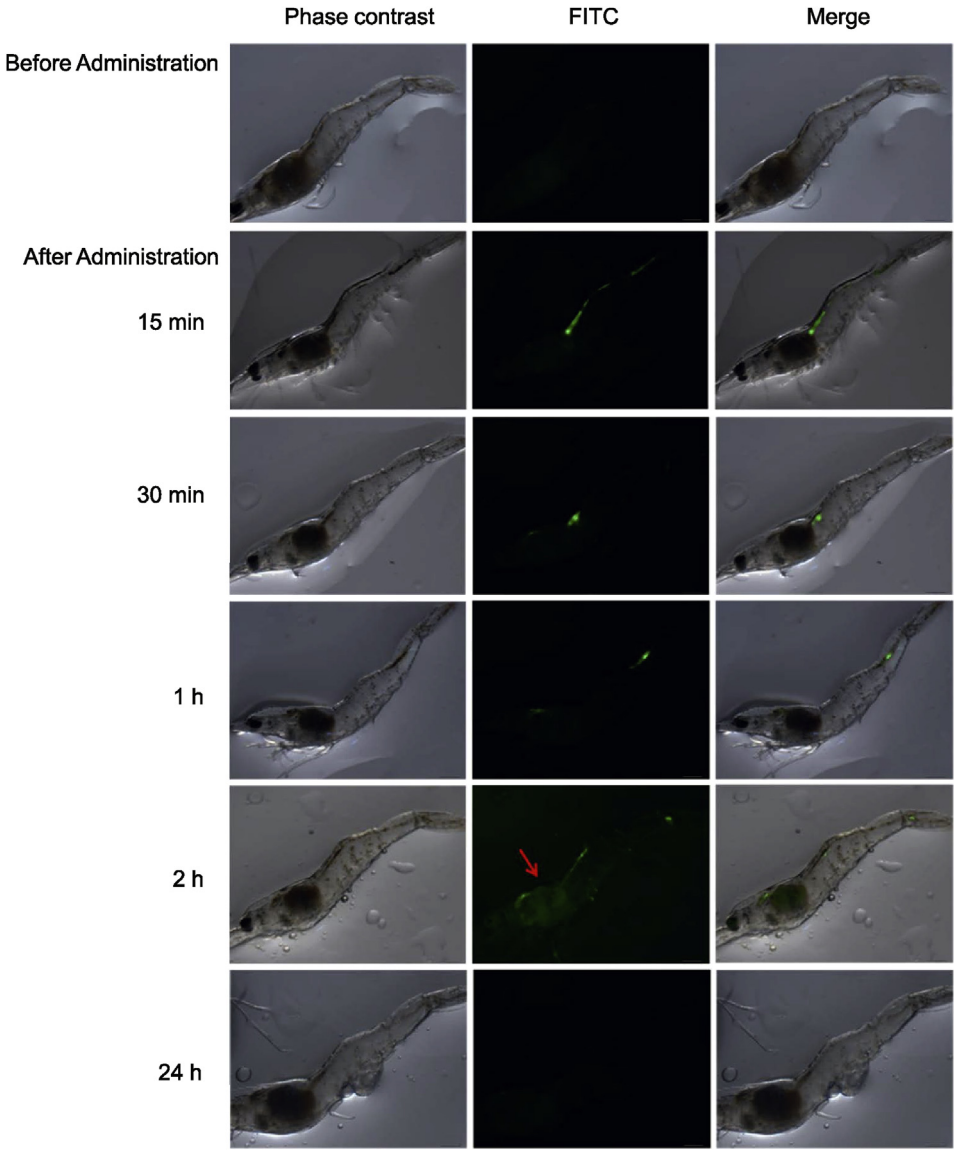

Fig. 4. Shrimp administered with *Artemia* bioencapsulated with FITC-SG (20 μg mL^{-1}) at different time points observed under a stereo-fluorescent microscope. The green fluorescence represented the FITC-SG in the digestive tract of shrimp. Scale bar = 5 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phenoloxidase, superoxide anions and superoxide dismutase activities [18]. Furthermore, we identified a potential immune stimulating mechanism for SG using a haemocyte culture model in which SG interacted with the shrimp haemocyte membrane protein, lipopolysaccharide and β-1,3-glucan binding protein (LGBP) followed by activation of the immune genes [17]. In the present study, we sought to investigate the capacity of the delivery of SG via the process of bioencapsulation in *Artemia* and to determine the optimal enrichment and efficacy duration of the immune enhancing effect of SG in shrimp in order to establish the most efficient feeding regimen.

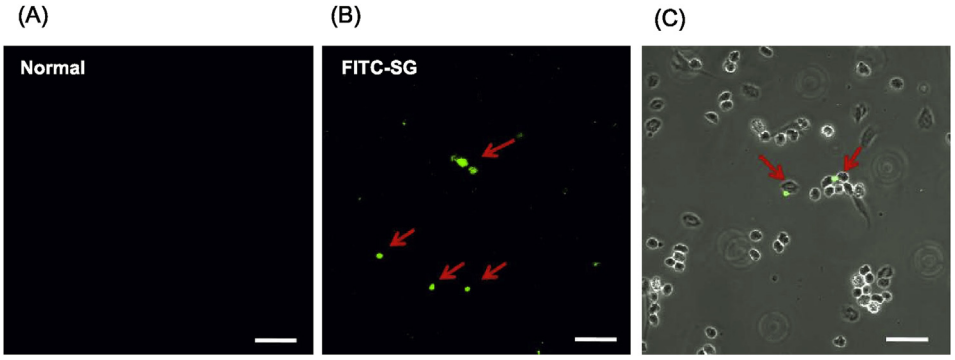

Fig. 5. Fluorescent micrographs showing the FITC-SG (green) in the haemolymph of (A) control shrimp, (B) SG administered shrimp. (C) Merged fluorescent and phase contrast micrograph showing haemocytes from the shrimp administered with SG bioencapsulated *Artemia* bound to FITC-SG (green). Scale bar = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

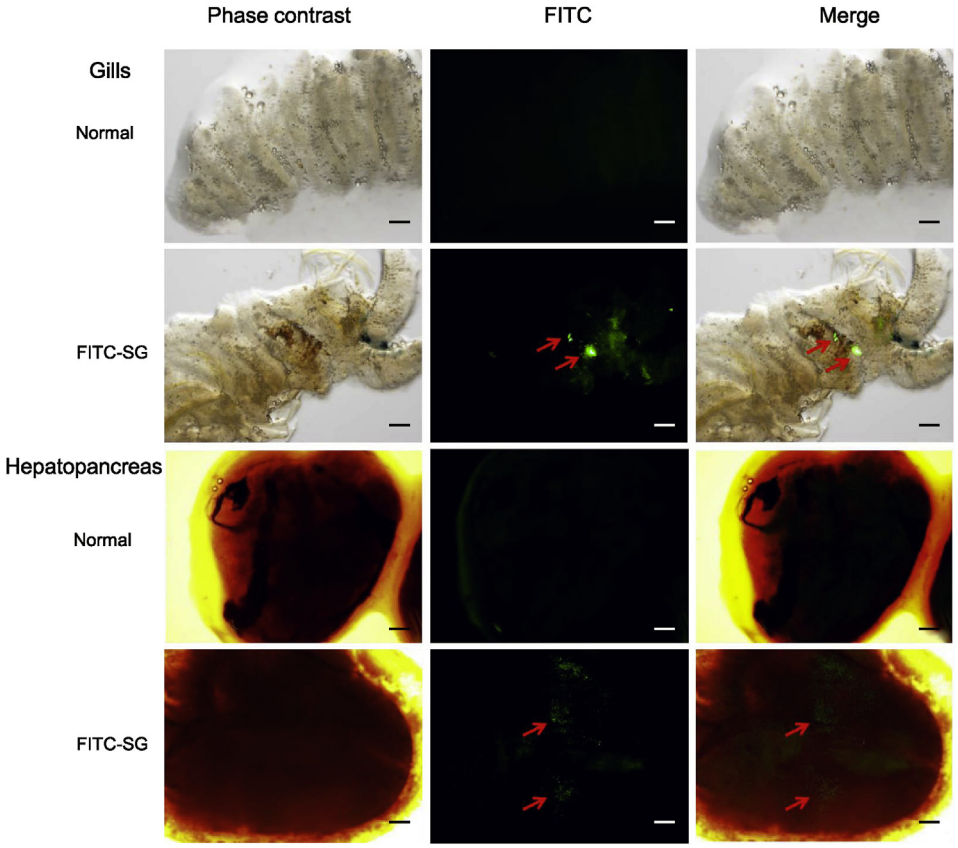

Fig. 6. Phase contrast, fluorescent and the merged micrographs of the shrimp tissues. FITC-SG (green) in gills and hepatopancreas of shrimp administered with *Artemia* bioencapsulated with FITC-SG observed under a stereo-fluorescent microscope. Merged micrographs showing FITC-SG in gills and FITC-SG distributed in hepatopancreas. Scale bar = 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

SG conjugated with FITC (FITC-SG) was employed for a visual monitoring of SG bioencapsulation in *Artemia*. Before enrichment, the toxicity of FITC-SG was evaluated. The result showed that FITC-SG (10–100 μg mL^{-1}) had no toxic effect on *Artemia* which was in agreement with our previous studies which found that SG at concentrations ranging from 10 to 2000 μg mL^{-1} caused no significant cytotoxicity in *Artemia* [18] and in haemocyte culture [25]. Studies in other cell types such as human pulmonary fibroblast cells revealed that SPs-FITC was non-toxic at concentrations ranging from 25 to 100 μg mL^{-1} [26]. To determine the optimal enrichment duration of SG in *Artemia*, a time-course observation of the fluorescein FITC-SG loading in the *Artemia* gut lumen was examined using a stereo-fluorescent microscope. We found that uptake of the FITC-SG in the gut lumen of the *Artemia* was time- and concentration-dependent. Optimal loading of SG in the gut lumen of the *Artemia* immersed in FITC-SG 20 μg mL^{-1} was reached at 6 h, with a gradual increase towards 24 h which was not significant. Enrichment of the *Artemia* with higher concentrations of FITC-SG for 6 h indicated that a maximum capacity of SG uptake in the *Artemia* was achieved at 40 μg mL^{-1}. Our results are in agreement with other studies. For instance, Immanuel et al. (2007) [27] reported that *Artemia* bioencapsulated with the fatty acid (HUFA) and probiotics needed a 6 h enrichment for 100% gut loading. Arulvasu et al. (2012) [28] showed that the optimum exposure duration for the leaf extract of *V. negundo* (at 2.5 mg mL^{-1}) in *Artemia* nuaplii was 12 h. In addition, Kanjana et al. (2011) [19] reported that an enrichment duration of 12 h was optimal for the bioencapsulation of the ethanol extract from *G. fisheri* in *Artemia*.

Our study demonstrates for the first time the tracking of FITC-SG bioencapsulated *Artemia* to the shrimp intestinal tract and tissues, and the delivery of SG by *Artemia* bioencapsulation to the circulation of the shrimp where it binds to haemocytes. Interestingly, Batel et al. (2016) [29] reported that microparticles tracked with fluorescence were transferred from *Artemia* to zebrafish intestinal tracts, intestinal epithelium and then distributed into other organs including gill and liver. In a previous study we reported that SG bound with LGBP and subsequently activated expression of the LGBP downstream signaling mediators and their immune related genes in shrimp haemocytes [17], along with the present study, suggesting that SG could pass from the GI tract and interact with haemocytes.

Recently, it was reported that feeding shrimp with *Artemia* bioencapsulated with SG at 100 and 200 μg mL^{-1} for 7 days enhanced several immune parameters including THC, PO, SOD and O_2^- activities and thereby induced shrimp resistance to white spot syndrome virus (WSSV) infection as shown by a significantly lower mortality rate and less viral load in the shrimp [18]. Furthermore, our present study demonstrated that the oral administration of *Artemia* bioencapsulated with SG (0.5 mg g^{-1} BW/day) for 7 days up-regulated the expression of immune related genes in post larvae shrimp. The feeding dose at 0.5 mg g^{-1} BW was calculated from the amount of SG in *Artemia* enriched with SG at 200 μg mL^{-1}. This dose level of SG was the same as that used in a previous feeding trial in which shrimp were fed with an SG supplemented feed diet (2% w/w) and where expressions of LGBP signaling proteins were activated [30]. These findings suggest that SG at 0.5 mg g^{-1} BW is an effective dose for stimulating the expression of immune related genes in shrimp.

The longevity effect of immunostimulants in aquatic animals continues to be a topic of concern [5,31]. Upon examination of the efficacy of SG bioencapsulation in *Artemia* to enhance shrimp immune activity, following a 3-consecutive-day administration, we found that the early up-regulated immune genes were IMD and IKKβ (1 day post SG) while the late up-regulated genes were dicer and proPO-I (3 days post SG). The levels of dicer and proPO-I were significantly higher than control (day 3–7 post SG) and remained high through day 14 post SG.

These findings were similar to previous studies, although different means of administration and animal stages were applied. For instance, when shrimp *L. vannamei* received alginate [32], carrageenan [33] and sulfated polysaccharide (SPs) from *G. tenuistipitta* [34] via injection there was an increase in respiratory burst and PO activity within 2–3

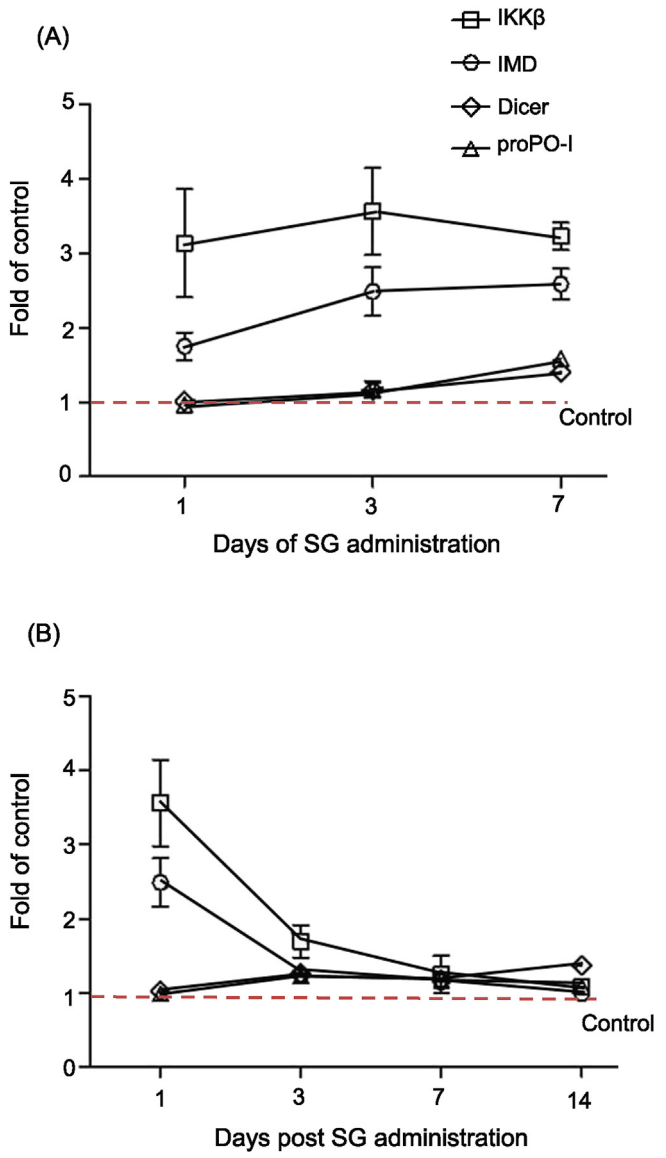

Fig. 7. Differential expression of the immune related genes (IKKβ, IMD, dicer and proPO-I genes) in shrimp administered with *Artemia* bioencapsulated with SG determined using RT-PCR. (A) The shrimp were daily administered with *Artemia* bioencapsulated with SG for 1, 3 and 7 days and then the samples collected to determine expressions of immune related genes. (B) The shrimp were administered with *Artemia* bioencapsulated with SG for a 3-consecutive day and the samples were collected on day 1, 3, 7 and 14 post SG administration, n = 15. All RT-PCR values were normalized against the constitutive gene, EF-1α, and the expression of a specified immune gene is presented as relative to control group (fold of control group).

days after injection which then gradually declined. The administration of *Sargassum* extract [35] or carrageenan [36] to *L. vannamei* by immersion increased immune parameters including haemocyte count, PO activity, respiratory burst, and lysozyme activity after 3 h, which showed a slight decline 5–6 h post administration. Indeed, it is accepted that the immunostimulant efficacy of SPs extracted from a particular seaweed depend on the source of seaweed, season of seaweed harvest, method of extraction, and shrimp species [37], which may actuate different changes of immune parameters in shrimp. For instance, treatment with 500 mg kg^{-1} fucodians (SPs from brown seaweed) in the diet increased phagocytic activity (17–40%) in shrimp *L. vannamei* [38], while the same concentration of fucoidans was able to increase the phagocytic activity in shrimp *P. monodon* by only 9.1% [39]. In another study of *L. vannamei* it was reported that fucoidans from different brown seaweed species produced differential levels of immune parameters and immune gene expression [38].

IMD and IKKβ are important upstream signaling mediators which regulate the expression of the immune response in shrimp [40]. The expression of IMD and IKKβ is mediated by activation of LGBP [40,41]. This leads to cleavage of the N-terminal RHD (NF-κB) of Relish which then translocates into the nucleus and immediately turns on the expression of antimicrobial peptides and antiviral genes (dicer) [41,42]. A similar activation of the NF-κB pathway occurs when LvIKKβ is overexpressed in *Drosophila S2* cells and HEK 293T cells which affects the expression of antimicrobial peptides such as PENs, lysozyme and crustins [42]. Our previous data has shown that when shrimp and shrimp haemocytes are treated with SG the IMD-IKKs–NF–κB signaling pathway is activated [17]. Data from the present study indicated an early increased expressions of IMD and IKKβ following bioencapsulated SG administration. This would likely activate the intermediate mediators like NF-κB which would then signal the expression of downstream immune genes, proPO-I and dicer. Moreover, our data shows that as the expression of IMD and IKKβ declines post administration the expression of proPO-I and dicer increases and is maintained.

In conclusion, this study demonstrates that SG encapsulated in *Artemia* migrates to target cells in different tissues and stimulates an immune response in shrimp. The efficacy of the 3-consecutive-day SG regimen is shown to be sufficient for boosting and maintaining the high expression levels of immune genes in post larvae shrimp.

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References

[1] D. Meena, P. Das, S. Kumar, S. Mandal, A. Prusty, S. Singh, et al., Beta-glucan: an ideal immunostimulant in aquaculture (a review), Fish Physiol. Biochem. 39 (2013) 431–457.
[2] E. Ringø, R.E. Olsen, J.L.G. Vecino, S. Wadsworth, S.K. Song, Use of immunostimulants and nucleotides in aquaculture: a review, J. Mar. Sci. Res. Dev. 2 (2012) 104.
[3] B. Petrunov, P. Nenkov, R. Shekerdjiisky, The role of immnostimulants in immunotherapy and immunoprophylaxis, Biotechnol. Biotechnol. Equip. 21 (2007) 454–462.
[4] P. Martínez Cruz, A.L. Ibáñez, O.A. Monroy Hermosillo, H.C. Ramírez Saad, Use of probiotics in aquaculture, ISRN Microbiol. (2012) 13.
[5] V.J. Smith, J.H. Brown, C. Hauton, Immunostimulation in crustaceans: does it really protect against infection, Fish Shellfish Immunol. 115 (2003) 71–90.
[6] J. Heppell, H.L. Davis, Application of DNA vaccine technology to aquaculture, Adv. Drug Deliv. Rev. 43 (2000) 29–43.
[7] A. Dey, K. Ghosh, N. Hazra, An overview on bioencapsulation of live food organisms with probiotics for better growth and survival of fresh water fish juveniles, Int. J. Res. Fish. Aquac. 5 (2015) 74–83.
[8] P. Sorgeloos, P. Dhert, P. Candreva, Use of the brine shrimp, *Artemia* spp., in marine fish larviculture, Aquaculture 200 (2001) 147–159.
[9] T. Watanabe, C. Kitajama, S. Fujita, Nutritional values of live organisms used in Japan for mass propagation of fish: a review, Aquaculture 34 (1983) 115–143.
[10] M.A. Cook, M.B. Rust, Bioencapsulation of five forms of erythromycin by adult *Artemia salina* (L.), J. Fish Dis. 25 (2002) 165–170.
[11] J. Gomes, C.L. Vilela, R. Bexiga, G.D. Nunes, N. Pereira, L.M. Cavaco, Fish antibiotherapy: bioencapsulation of flumequine using adult brine shrimp (*Artemia salina*), Aquacult. Res. 38 (2007) 613–617.
[12] R. Campbell, A. Adams, M.F. Tatner, M. Chair, P. Sorgeloos, Uptake of *Vibrio anguillarum* vaccine by *Artemia salina* as a potential oral delivery system to fish fry, Fish Shellfish Immunol. 3 (1993) 451–459.
[13] F.J. Gatesoupe, Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic vibrio, Aquat. Living Resour. 7 (1994) 277–282.
[14] T.J. Majack, M.B. Rust, K.C. Massee, G.W. Kissil, R.W. Hardy, M.E. Peterson, Bioencapsulation of erythromycin using adult brine shrimp, *Artemia franciscana* (Latreille), J. Fish Dis. 23 (2000) 71–76.

[15] M.C. Allender, M. Kastura, R. George, F. Bulman, J. Yarborough, S. Cox, Bioencapsulation of praziquantel in adult *Artemia*, J. Bioanal. Biomed. 2 (2010) 96–99.
[16] M.C. Allender, M. Kastura, R. George, F. Bulman, J. Yarborough, S. Cox, Bioencapsulation of metronidazole in adult brine shrimp (*Artemia sp.*), J. Zoo Wildl. Med. 42 (2011) 241–246.
[17] T. Rudtanatip, B. Withyachumnarnkul, K. Wongprasert, Sulfated galactans from *Gracilaria fisheri* bind to shrimp haemocyte membrane proteins and stimulate the expression of immune genes, Fish Shellfish Immunol. 47 (2015) 231–238.
[18] 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 (2014) 52–60.
[19] K. Kanjana, T. Radtanatip, S. Asuvapongpatana, B. Withyachumnarnkul, K. Wongprasert, Solvent extracts of the red seaweed *Gracilaria fisheri* prevent *Vibrio harveyi* infections in the black tiger shrimp *Penaeus monodon*, Fish Shellfish Immunol. 30 (2011) 389–396.
[20] C. Seenivasan, P. Saravana Bhavan, S. Radhakrishnan, R. Shanthi, Enrichment of *Artemia nauplii* with *Lactobacillus sporogenes* for enhancing the survival, growth and levels of biochemical constituents in the postlarvae of the freshwater prawn *Macrobrachium rosenbergii*, Turk. J. Fish. Aquat. Sci. 12 (2012) 23–31.
[21] T. Tanaka, Y. Fujishima, S. Hanano, Y. Kaneo, Intracellular disposition of polysaccharides in rat liver parenchymal and nonparenchymal cells, Int. J. Pharm. 286 (2004) 9–17.
[22] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[23] Y. Kajita, M. Sakai, S. Atsuta, M. Kobayashi, The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*, Fish Pathol. 25 (1990) 93–98.
[24] C. Langdon, A. Nordgreen, M. Hawkyard, K. Hamre, Evaluation of wax spray beads for delivery of low-molecular weight, water-soluble nutrients and antibiotics to *Artemia*, Aquaculture 284 (2008) 151–158.
[25] T. Rudtanatip, S. Asuvapongpatana, B. Withyachumnarnkul, K. Wongprasert, Sulfated galactans isolated from the red seaweed *Gracilaria fisheri* target the envelope proteins of white spot syndrome virus and protect against viral infection in shrimp haemocytes, J. Gen. Virol. 95 (2014) 1126–1134.
[26] X. Du, S. Jiang, H. Liu, X. Xin, J. Li, M. Geng, et al., MS80, a novel sulfated polysaccharide, inhibits CD40-NF-kappaB pathway via targeting RIP2, Mol. Cell. Biochem. 337 (2010) 277–285.
[27] G. Immanuel, T. Citarasu, V. Sivaram, V. Selva Shankar, A. Palavesam, Bioencapsulation strategy and highly unsaturated fatty acids (HUFA) enrichment in *Artemia franciscana* nauplii by using marine trash fish *Odonus niger* liver oil, Afr. J. Biotechnol. 6 (2007) 2043–2053.
[28] C. Arulvasu, S. Shobana, H.A.A. Banu, D. Chandhirasekar, D. Prabhu, Bioencapsulation of *Artemia* nauplii with herbal extract for promoting growth of fish fry *Poecilia sphenops val*, J. Mod. Biotechnol. 1 (2012) 37–44.
[29] A. Batel, F. Linti, M. Scherer, L. Erdinger, T. Braunbeck, Transfer of benzo[a]pyrene from microplastics to *Artemia nauplii* and further to zebrafish via a trophic food web experiment: CYP1A induction and visual tracking of persistent organic pollutants, Environ. Toxicol. Chem. 35 (2016) 1656–1666.
[30] T. Rudtanatip, N. Boonsri, S. Asuvapongpatana, B. Withyachumnarnkul, K. Wongprasert, A sulfated galactans supplemented diet enhances the expression of immune genes and protects against *Vibrio parahaemolyticus* infection in shrimp, Fish Shellfish Immunol. 65 (2017) 186–197.
[31] R.A. Dalmo, J. Bøgwald, β-glucans as conductors of immune symphonies, Fish. Shellfish Immunol 25 (2008) 384–396.
[32] W. Cheng, C.H. Liu, S.T. Yeh, J.C. Chen, The immune stimulatory effect of sodium alginate on the white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*, Fish Shellfish Immunol. 17 (2004) 41–51.
[33] S.T. Yeh, J.C. Chen, Immunomodulation by carrageenan in the white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*, Aquaculture 276 (2008) 22–28.
[34] W.Y. Hou, J.C. Chen, The immunostimulatory effect of hot-water extract of *Gracilaria tenuistipitta* on the white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*, Fish Shellfish Immunol. 19 (2005) 127–138.
[35] T.G. Huynh, S.T. Yeh, Y.C. Lin, J.F. Shyu, L.L. Chen, J.C. Chen, White shrimp *Litopenaeus vannamei* immersed in seawater containing *Sargassum hemiphyllum* var. *chinense* powder and its extract showed increased immunity and resistance against *Vibrio alginolyticus* and white spot syndrome virus, Fish Shellfish Immunol. 31 (2011) 286–293.
[36] 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 (2014) 352–366.
[37] A. Robic, J.F. Sassi, P. Dion, Y. Lerat, M. Lahaye, Seasonal variability of physicochemical and rheological properties of ulvan in two Ulva species (Chlorophyta) from the Brittany coast, J. Phycol. 45 (2009) 962–973.
[38] A. Setyawan, A. Isnansetyo, Murwantoko, S. Indarjulianto, C.R. Handayani, Comparative immune response of dietary fucoidan from three Indonesian brown algae in white shrimp Litopenaeus vannamei, AACL Bioflux 11 (2018) 6.
[39] W. Chotigeat, S. Tongsupa, K. Supamataya, A. Phongdara, Effect of fucoidan on disease resistance of black tiger shrimp, Aquaculture 233 (2004) 23–30.
[40] A. Tassanakajon, K. Somboonwiwat, P. Supungul, S. Tang, Discovery of immune molecules and their crucial functions in shrimp immunity, Fish Shellfish Immunol. 34 (2013) 954–967.
[41] Y. Chen, X. Li, J. He, Recent advances in researches on shrimp immune pathway involved in white spot syndrome virus genes regulation, J. Aquac. Res. Dev. 5 (2014) 228.
[42] P.H. Wang, Z.H. Gu, D.H. Wan, B.D. Liu, X.D. Huang, S.P. Weng, The shrimp IKK–NF-kB signaling pathway regulates antimicrobial peptide expression and may be subverted by white spot syndrome virus to facilitate viral gene expression, Cell. Mol. Immunol. 10 (2013) 423–436.