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Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)
&
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http://www.aquaculturehub.org

ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_68.2016.1289, 13 pages

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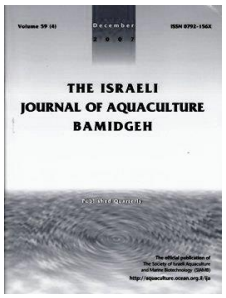

Effect of *Sargassum oligocystum* Hot-Water Extract on Innate Immune Response and Survival of Summer Flounder *Paralichthys dentatus* to *Vibrio harveyi* Challenge

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Keywords: seaweed hot-water extract, non-specific immune response, *Paralichthys dentatus, Sargassum oligocystum, Vibrio harveyi*

Abstract

The present study evaluated the effects of hot-water extract of a brown seaweed *Sargassum oligocystum* on the non-specific immune response and survival of summer flounder *Paralichthys dentatus,* to *Vibrio harveyi* bacterial challenge. Fish were either immersed in 100 or 500 mg/L or injected with 0.1 or 0.5 mg per fish of the hot-water extract. The innate humoral (lysozyme, plasma protein, bactericidal activity), cellular (respiratory burst, hematocrit), and disease resistance to *Vibrio harveyi* infection were determined. Results showed that all the experimental treatments either by injection or immersion, significantly enhanced respiratory burst activity and hematocrit values of the fish. In the experiment, all the treatments of hot-water extract significantly affected the lysozyme, plasma protein, and bactericidal activity of the experimental fish from days 1 to 5 after delivery. Following the bacterial challenge mortality decreased significantly in all treated groups. In summer flounder, administration of hot-water extract of *S. oligocystum* either by injection or immersion was found to be an immunoprophylactic for finfish aquaculture. The efficacy of using hot-water extract as a feed supplement or feed additive needs further examination.

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Introduction

Conditions in aquaculture often lead to overcrowding and impaired water quality and this in turn leads to stress in cultured fish. Chronic stress adversely affects fish health, resulting in inhibition of specific immune responses and defense mechanisms which leads to increased susceptibility to infections. Traditionally, synthetic chemicals and antibiotics have been used as preventive or prophylactic means of treating fish diseases. However, emergence of antibiotic-resistant microorganisms has reduced the effectiveness of synthetic chemicals and antibiotics.

Summer flounder *Paralichthys dentatus,* is a commercially valuable species of flatfish found in the Northwest Atlantic and also along the east coast of the United States (Fishbase). Declining wild stock and subsequent commercial fishing quota restrictions (NOAA/ NFMA, 1993) have led to the development of commercial culture (Bengtson, 1999; Schwarz, 2003). Disease is a constraint in the culture of summer flounder. An epizootic at a grow-out facility in Rhode Island, United States led to initial reports and identification of Flounder Infectious Necrotizing Enteritis (FINE), a disease caused by *Vibrio harveyi* (Soffientino et al., 1999; Gauger and Gomez-Chiarri, 2002). This disease continues to affect summer flounder culture facilities in the Northeast U.S. (Gauger and Gomez-Chiarri 2006).

Use of immunostimulants in aquaculture has grown as they are effective in increasing host immunity and preventing disease outbreaks (Kim et al., 2012). Immunostimulants which include substances of microbial origin such as polymers, glucans, and lipopolysaccahrides, vitamins, or synthetic compounds such as levamizole and hydroxyl-methyl-butyrate, as well as extracts from animals, terrestrial plants, and marine organisms such as seaweeds (Ganguly, 2010) are a heterogeneous group of compounds which stimulate immune systems. Most immunostimulants boost innate defense mechanisms and may have positive effects on antibody synthesis. Some studies have shown that immunostimulants can protect fish against bacterial pathogens. A wide range of immunostimulants have been used in aquaculture to improve growth and resistance to pathogens (Dalmo and Bogwald, 2008; Kunttu et al., 2009).

Immunostimulants can be easily applied to small fish, and the application can be scheduled when disease outbreaks are expected, such as prior to transport of juvenile summer flounder from the hatchery to grow out facilities, after which outbreaks of flounder infectious necrotizing enteritis (FINE) most commonly occur (Soffientino et al., 1999; Gauger and Gomez-Chiarri 2006).

Several studies have shown that various substances derived from seaweeds, mainly polysaccharides, can modify immune response and increase protection against infectious diseases in finfish.

The aim of this study was to determine the potential of hot-water extract of *S. oligocystum*, brown algae from Cagayan, Philippines, as an immunostimulant, by examining the effect on immune parameters of summer flounder. These parameters include total hematocrit (Hct), respiratory burst assay (RBA), plasma lysozyme, plasma protein, bactericidal assay, as well as resistance of summer flounder to challenge with the bacterial pathogen *V. harveyi* DN01.

Materials and Methods

Experimental animals. The protocol for these experiments was approved by the Institutional Animal Care and Use Committee of the University of Rhode Island. Around 300 summer flounder *Paralichtys dentatus* (mean ± SE total length 21.37 ± 4.4 cm, mean ± SE body weight 98.3 ± 3.7 g) obtained from experimental tanks in the aquarium building of University of Rhode Island (URI) Narragansett Bay campus. These were transported to the Blount Aquaculture Laboratory Building. The fish were randomly distributed into twelve, 50 gallon aquaria (12 fish per aquarium) within a flow-through system. Temperature, salinity, and dissolved oxygen of the seawater during the experimental period were 18-20 ºC, 33-34 ‰, and 5.6-6.2 ppm, respectively. The fish were fed twice daily (1000 H and 1600 H) to satiation using prepared diets, and acclimated for two weeks prior to start of the experiment. A subsample of 10 fish were

also examined and cleared for the presence of pathogens or other pathological conditions.

Bacteria. An inoculum of *Vibrio harveyi* DNO1 (Soffientino et al. 1999) from stock cultures stored in LB20 with 20% glycerol at -80°C was plated on LB20 (Luria-Bertani media with 20% NaCl) agar and maintained at room temperature. Pathogenicity of the bacteria was confirmed through three passage assays in summer flounder (Gauger and Gomez-Chiarri 2006). For the challenge experiments, the bacteria were re-inoculated in LB20 broth and incubated in a shaker for 24 h at 26°C prior to use. The culture was harvested by centrifugation at 2500 g for 15 min. The concentrated cells were washed three times with Phosphate Buffered Saline (PBS) at pH 7.2 and re-suspended into PBS. A preliminary LD50 experiment was conducted to determine the appropriate challenge dose of the bacteria to be used for the study (Soffientino et al,. 1999).

Preparation of the hot-water extract of S. oligocystum. S. oligocystum was collected from the coastal area of Sta. Ana, Cagayan, Philippines. The hot-water extract of *S. oligocystum* was prepared based on the method described by Fujiki et al. (1992) and Hou and Chen (2005). Ten gram of dried *S. oligocystum* powder was added to 250 ml of deionized distilled water and boiled for 3 h. The boiled suspension was passed through muslin cloth and the filtrate frozen at -80°C for 24h and lyophilized under reduced pressure for 48 h. The 10 g of *S. oligocystum* dried powder yielded a harvest weight of 2.93 g after hot-water extraction.

Experimental treatments. Six treatments, three immersion treatments, and three inoculation treatments were tested. 3 concentrations of hot-water extract (HWE, 0 (control), 100, 500 mg/L) were tested by immersion of fish for 3h, and 3 inoculation treatments, 100 µl of PBS; 1 mg/l HWE; and 5 mg/ml HWE, at a final dose of 0.1 or 0.5 mg per fish) were delivered by intraperitoneal injection. Fish to be injected were anesthetized by immersion in seawater containing 100 mg/L tricane-s methane sulfonate (MS 222). All the treatments were applied in triplicate with 24 fish per treatment. After injection or immersion, fish were immediately transferred to glass aquaria containing 20 L of 34‰ aerated recirculated seawater. Each aquarium was fitted with individual filter units and covered with black plastic on each side to maintain a calmer environment for the flounder. Nets placed at the top of each aquarium prevented escape of fish. Temperature, dissolved oxygen, and salinity were maintained at 16-18ºC, 5.8-6.7 mg/L and 33.5-35 ‰, respectively, for the duration of the experiment.

Blood collection and plasma separation. Six fish were randomly selected from each treatment at days 1, 3, 5, and 7 post-administration of HWE. To minimize handling stress, the fish were anesthetized by immersion in water containing 100 mg/L MS222. Whole blood (1 ml) was collected from the caudal peduncle region of each fish using syringes (1 ml) with 25 gauge needles rinsed with 0.2 M ethylenediaminetetraacetic acid (EDTA) as anticoagulant. A portion of the dorsal fin of *P. dentatus* was cut after blood collection for identification purposes. The collected blood was transferred in 1.5 ml Eppendorf tubes rinsed with the same anticoagulant and kept on ice. Aliquots of blood for the hematocrit and respiratory burst assays were transferred to the respective containers. The rest of the blood was centrifuged at 6000 x g for 20 min to separate the plasma. Collected plasma was stored at -20ºC until use.

Plasma lysozyme activity. Lysozyme activity was determined using a turbidometric assay (El-Boshy, 2010). Chicken egg white lysozyme (Sigma, St. Louis, MO, USA) was used as standard and 0.75 mg/ml of *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA) was dissolved in 0.1 M sodium phosphate/citric acid buffer, pH 5.8, as substrate. Fifty microliters of plasma or standard were placed in triplicate wells in a 96-well microtiter plate, 150 µl substrate solution was added to each well, and plates were incubated at 26 ºC. The reduction in the absorbance at 450 nm was read after 30 sec and 20 min with a BioTek (BioTek Synergy HT, VT, USA) microplate reader. One unit of lysozyme activity was expressed as the reduction in the absorbance of 0.001/ min.

Plasma protein content. Protein content of the plasma was determined following the Coomassie (Bradford) Protein Assay (Bradford, 1976). Each plasma sample was diluted 50x with PBS. Ten microliters of the diluted plasma or standard (prepared with bovine

serum albumin, BSA) were added to the wells of flat-bottomed 96-well microtiter plate in triplicates. Two hundred microliters of diluted (4:1) Bio-Rad (Bio-Rad, Hocules, CA, USA) Coomassie blue protein assay dye was then added to each well. The samples and standards were allowed to incubate for 10 min at room temperature. Absorbance of the samples and standards were read at 595 nm using BioTek (BioTek Synergy HT, VT, USA) microplate reader. Plasma protein content was expressed as mg/ml of protein in the sample.

Plasma bactericidal activity. Plasma bactericidal activity was determined following the methods of El-Boshy (2010). 40 µl of plasma or Hank's Balanced Salt Solution (HBSS) (positive control) and 100 µl of a 24 h culture of *V. harveyi* in LB20 media were added in triplicates to the wells of a 96-round bottom microtiter plate and incubated for 2.5 h. Following incubation, 25 µl of 3-(4,5 dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide (MTT; 2.5 mg/ml) (Sigma, St. Louis, MO, USA) was added to the wells and the plate was incubated at room temperature for 10 min to allow the formation of formazan. The plate was then centrifuged for 10 min at 3200 rpm. The supernatant was discarded and the precipitate was dissolved in 200 µl of DMSO. The absorbance of the dissolved formazan was read at 560 nm. Bactericidal activity was calculated as the decrease in the number of viable *V. harveyi* cells by subtracting the absorbance of samples from that of the control (HBSS) and reported as absorbance units.

Hematocrit. Hematocrit micro capillary tubes were filled to two-thirds with whole blood and sealed, and centrifuged at 12000 g for 5 min. Total hematocrit of the blood samples was obtained by determining the percentage of packed cell volume (PCV) using an hematocrit tube reader.

Respiratory burst assay. Respiratory burst assay of whole blood was determined using the method of Anderson and Siwicki, 1995 with some modifications. 50 µl of whole blood was placed in triplicate in wells of flat-bottomed 96-well microtiter plates. The plate was then incubated for 1h at 16 ºC. The supernatant was removed and the wells were rinsed three times with PBS. Fifty microliters of activated DCF [dichloroflourescin; 2',7'-dihydrodichloroflourescin-di-acetate, H2DCFDA (Invitrogen, USA), was converted to DCF by adding 0.5 ml of 1 mM H2DCFDA in ethanol to 2.0 ml 0.01 N NaOH and allowed to stand at room temperature for 30 min, then neutralized with 10 ml of 25 mM sodium phosphate buffer, and stored at -20 ºC] was added. Then 50 µl of Phorbol Myristate Acetate [PMA, Sigma, St. Louis, MO, USA; dissolved in DMEM-F12 (Sigma, St. Louis, MO, USA) was added at a stock concentration of 108.11 µg/ml and stored in the dark at -20ºC until use]. The plates were placed in a spectrofluorometer (BioTek Synergy HT, VT, USA), excited at 480 nm, and emission was read at 530 nm immediately and after 1 h of incubation at room temperature. Respiratory burst activity was expressed as the fluorescence units after 1h exposure to DCF and PMA.

Bacterial challenge experiment. Twenty fish from each treatment group (10 fish per replicate) were transferred to glass aquaria at the Blount Pathology room on day 9 post administration of the HWE. Juvenile summer flounder were experimentally challenged with *V. harveyi* isolate DN01. Bacterial solutions were prepared by growing cultures in LB20 overnight at room temperature, collecting the cells by centrifugation (3000×g, 5–10 min), and washing thrice with PBS. Bacterial concentrations were determined by measuring the optical density at 490 nm (OD490) and comparing values to a standard curve made by plotting optical density (OD490) versus plate counts on LB20 plates. For the challenge experiments, 10 fish were anesthetized in MS-222 (Sigma) and inoculated by intraperitoneal (IP) injection of 100 µl of 5.85 x 107 cells/ml per fish in PBS (infected group, all isolates) or sterile PBS (negative controls). After inoculation, fish were held in 20 L glass aquaria equipped with filters. Aeration was provided using air stones. Fish were observed twice daily for 10 d for lesions and mortalities. *V. harveyi* was re-isolated from the organs of the moribund fish to confirm that mortality was caused by bacterial infection.

Statistical analysis. The effect of treatment on immune parameters was evaluated using a two-way ANOVA, with treatment and days as variables. A multiple-comparison (Tukey's) test was used to determine significant differences among treatments using the

SAS computer software (SAS Institute, Cary, NC, USA). Statistical significance of differences required that $p<0.05$.

Results

Effect of HWE on humoral innate immune responses.

Plasma lysozyme. Plasma lysozyme was significantly higher in the fish that received *S. oligocystum* HWE via injection with 1 or 5 mg/ml or immersion at 100 or 500 mg/L, than the control groups (0 mg/L and PBS injected) at 3-7 days post administration (dpa) of HWE (Fig. 1). Highest lysozyme activity was observed on day 7 in fish immersed in 500 mg/L of HWE, followed by fish immersed in 100 mg/L (329.28 units ml/L and 270.98 units ml/L, $p<0.001$ compared to the respective control) and on day 3 in the fish immersed in 500 mg/L HWE (264.64 units ml/L, $p<0.001$ compared to control). No statistical differences in lysozyme levels were observed between fish treated with HWE by either immersion or injection.

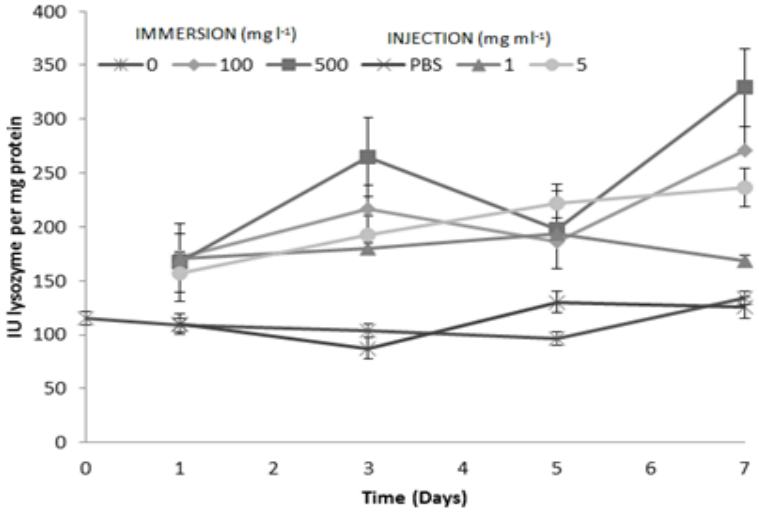

Fig. 1. Plasma lysozyme levels of summer flounder *Paralichthys dentatus* treated with a hot water extract of *Sargassum oligocystum* by immersion or injection. Values are presented as the mean (n=6) ± SE. Data at the same time point (day) with different letters are significantly different ($p < 0.05$) among treatments.

Plasma protein. Treatment of fish with HWE by immersion or injection led to a significant increase in plasma protein levels relative to the control at days 3 (all treatments with the exception of the 0.5 mg per fish injection, $p<0.001$) and 7 (for the fish immersed in 500 mg/L and the fish injected with 0.1 mg) post-administration of the HWE (Fig. 2).

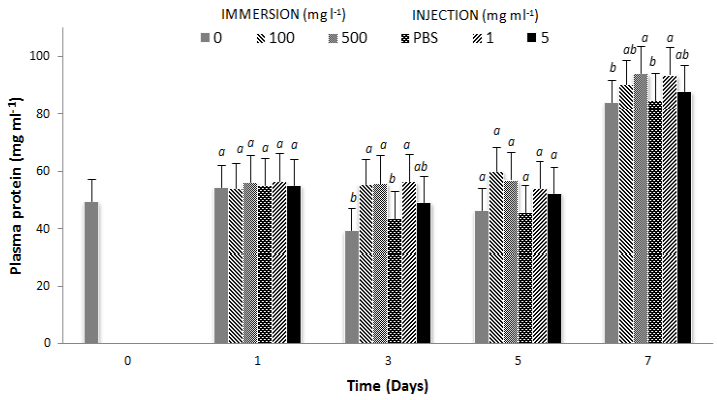

Fig. 2. Plasma protein content of summer flounder *Paralichthys dentatus* exposed to a hot water extract of Sargassum oligocystum by immersion or injection. Values are presented as the mean (n=6) ±SE. Data at the same time point (day) with different letters are significantly different ($p < 0.05$) among treatments.

Plasma bactericidal activity. Significantly higher bactericidal activity compared to the control was observed in the plasma of fish immersed in 500 mg/L HWE of S. oligocystum (1, 5 and 7 dpa) and fish injected with 0.1 and 0.5 mg of HWE (1 dpa only) (Fig. 3).

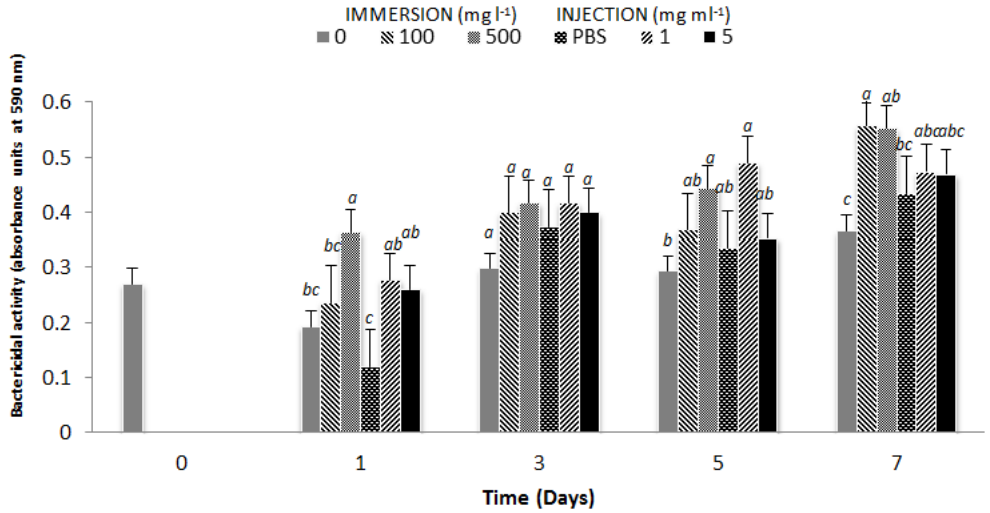

Fig. 3. Bactericidal activity in plasma of summer flounder *Paralichthys dentatus* exposed to a hot water extract of *Sargassum oligocystum* by immersion or injection. Values are presented as the mean (n=6) ±SE. Data at the same time point (day) with different letters are significantly different ($p < 0.05$) among treatments.

Effect of S. oligocystum HWE on cellular innate immune responses

Hematocrit. The hematocrit of fish immersed at 100 or 500 mg/L HWE of S. oligocystum was significantly higher than that of the control fish in most days tested (Fig. 4). On the other hand, exposure of fish to HWE by injection only led to a significant increase in hematocrit compared to control for the lowest dose (0.1 mg per fish) at 5 and 7 dpa.

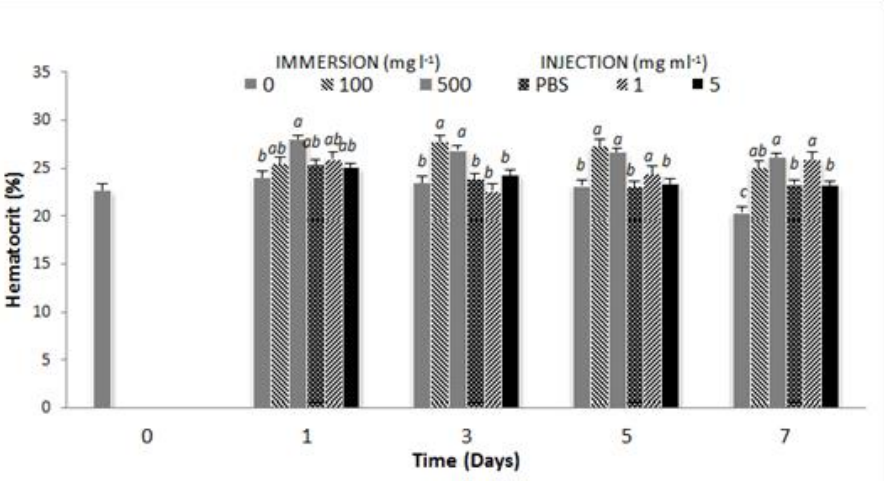

Fig. 4. Hematocrit values of summer flounder *Paralichthys dentatus* that received the hot water extract of *Sargassum oligocystum* by immersion or injection. Values are presented as the mean (n=6) ±SE. Data at the same time point (day) with different letters are significantly different ($p < 0.05$) among treatments.

Respiratory burst activity. Exposure of fish to the *S. oligocystum* HWE led to a significant increase in respiratory burst activity for most treatments (Fig. 5). It can be observed that the RB activity of the fish immersed at 100 or 500 mg/L HWE of *S. oligocystum* is higher than the fish injected with 1 or 5 mg/ml HWE on day 1 and day 3 post administration of immunostimulant. However, on day 5 and 7 post-administration of

HWE, the RB activity of fish injected with 1 or 5 mg/ml was significantly elevated. This is statistically different from the fish that received 100 or 500 mg/L HWE of *S. oligocystum* via immersion, and the control groups. Increased RB activity was observed on day 5 and 7 post-administration of HWE via injection with 1 or 5 mg/ml, or immersion at 100 or 500 mg/L HWE of *S. oligocystum*.

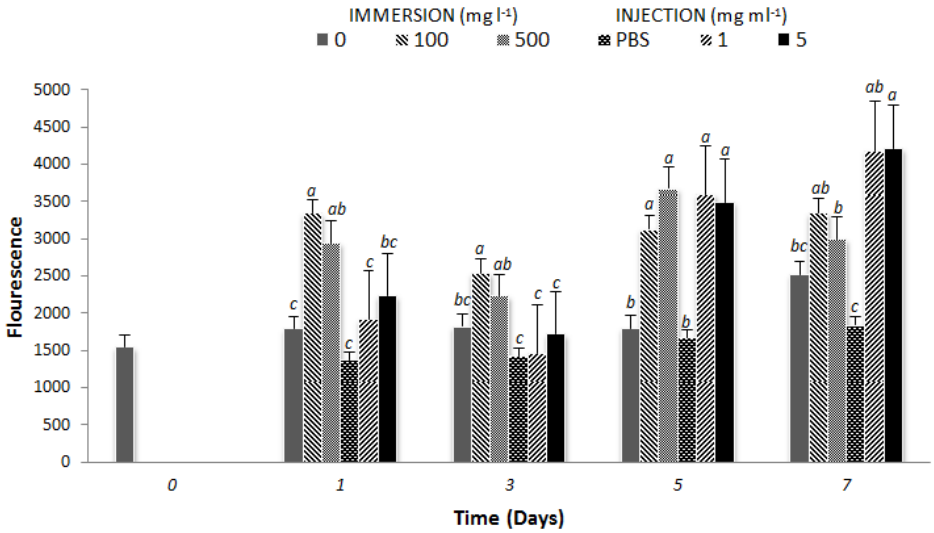

Fig. 5. Respiratory burst activity of summer flounder *Paralichthys dentatus* exposed to a hot water extract of *Sargassum oligocystum* by immersion or injection. Values are presented as the mean (n=6) ± SE. Data at the same time point (day) with different letters are significantly different ($p<0.05$) among treatments.

Effect of S. oligocystum HWE on survival to bacterial challenge

Survival in fish. Survival in fish exposed to HWE of *S. oligocystum* via immersion or injection and then challenged with *V. harveyi* 9 d post exposure to the HWE, was significantly higher than the control fish from days 4 to 10 after challenge (Fig. 6). Mortalities began in the control group (no HWE, injected with *V. harveyi*) on day 1 post-challenge, and all fish died in the two control groups on day 7 post-infection. At day 2 post-challenge with *V. harveyi* DN01, fish in the control groups began showing characteristic signs of flounder infectious necrotizing enteritis (FINE), including abdominal swelling, ascites, and the presence of blind-sac gut (colonic atresia). Confirmatory tests using API kit revealed that the bacteria isolated from the mucus and kidney of the infected fish was *V. harveyi*.

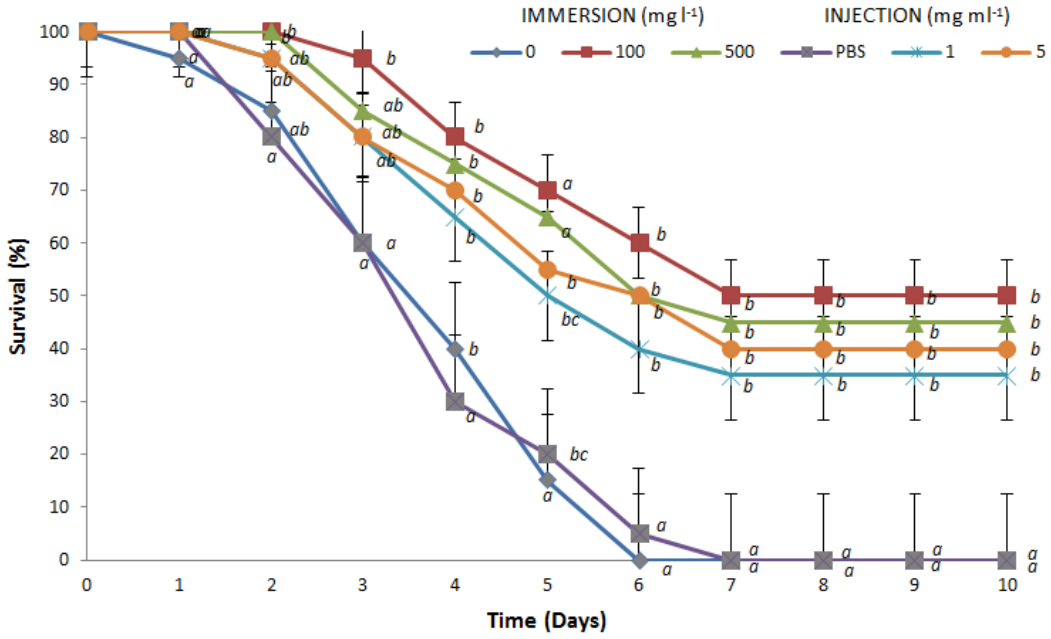

Fig. 6. Survival of the summer flounder *Paralichthys dentatus* that received the hot water extract of *Sargassum oligocystum* by injection or immersion and then challenged with *V. harveyi* DNO1. Values are presented as the mean ± SE.

Discussion

In this study we have shown that hot-water extract of brown seaweed *Sargassum oligocystum* increased survival and innate immune responses to bacterial challenge in a marine carnivorous flatfish species, summer flounder. The immunostimulatory effect of the hot-water extract was evident as early as 1 day post administration via immersion or injection, and persisted for at least 9 days, conferring significant protection against bacterial challenge at this point.

The innate immune system of fish is the first line of defense against invading pathogens (Narnaware et al., 1994). The major components of the immune system are macrophages, monocytes, granulocytes, and humoral elements such as lysozyme or complement system (Secombes and Fletcher, 1992; Magnadóttir, 2006). Immunostimulants from seaweed and seaweed extracts increase resistance to disease by enhancing non-specific defense mechanisms (Bhuvaneswari and Balasundaram,2006); Cheng et al., 2007; Cheng et al., 2008; Huang et al, 2006) and are widely used to improve impaired immune functions (Jeney et al., 1997; Sahoo et al., 2001). These products activate several components of the immune system, such as phagocytes, natural killer cells, T lymphocytes, B lymphocytes, complement and lysozyme (Anderson, 1992). Thus, immunostimulants offer a promising alternative to antibiotics, chemicals and vaccines.

Results obtained from the different tests have shown that the immune system of summer flounder responded to a hot-water extract of *S. oligocystum*, resulting in the enhancement of the immune system as indicated by the elevated levels of plasma lysozyme, total protein, and bactericidal activity, hematocrit, and respiratory burst activity, as well as a significant protection against bacterial challenge.

Lysozyme activity is one of the measurable components of non-specific defense mechanisms. Lysozyme is a cationic enzyme that hydrolyzes β-1-4-glucosidic linkages between N-acetyl muramic acid and N-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of a variety of bacterial pathogens (Alexander et al., 2010). Lysozyme, widely distributed in serum, tissues and mucus, is effective in lysis of not only Gram positive, but also Gram negative bacteria. In addition lysozyme also acts as an

opsonin and promotes phagocytosis (Yano, 1996). Previous studies have shown that immunostimulants, vaccines, and probiotics can enhance plasma lysozyme activity (Hanif et al., 2005; Kim and Austin, 2006). Lysozyme activity was reported to have been enhanced through oral administration of Astragalus radix root preparation in *Oreochromis niloticus* (Yin et al., 2006), *Astragalus* aspera seeds in *Labeo rohita* (Rao et al., 2006), a macro algae *Duraciella salina* in *Onchorhynchus mykiss* (Amar et al., 2004) and with various Chinese herbal seeds in Crussian carp (Chen et al., 2003), large yellow croaker (Jian and Wu, 2003) and common carp (Jian and Wu, 2004).

Plasma protein levels are indicative of increases in humoral elements of the immune system, e.g., immunoglobulins, transferrin, agglutinin, or precipitins (Magnadóttir, 2006). In this study, significant differences on plasma protein were observed only 7 dpa of the *S. oligocystum* HWE. In rainbow trout, the plasma total protein levels significantly increased after feeding fish with various herbal extracts (Dügenci et al., 2003).

Bactericidal activity of the serum has been well recognized as one of the key mechanisms of clearing bacteria in fish (Ellis, 2001). Bactericidal and bacteriolytic activity has been reported in several fish species including salmonids. It is present in the plasma and body fluids, and has also been detected in the mucus of rainbow trout (Harrell et al., 1976). In the marine teleost gilthead seabream, a significant enhancement of serum complement activity was found after feeding a 500 mg/kg levamisole-containing diet for 10 weeks (Mulero et al., 1998). This study demonstrated that the bactericidal capability of flounder was significantly elevated with the use of immunostimulants either by injection or immersion.

The ability of leucocytes to kill pathogenic microbes through respiratory burst is one of the most important protection mechanisms. Reactive oxygen and nitrogen are considered to be toxic for fish and bacterial pathogens (Miyazaki, 1998). Reactive oxygen produced by activated neutrophils and macrophages is capable of destroying invading pathogens and is considered an important indicator of non-specific defense in fish. Enhanced respiratory burst activity was also observed in fish treated with other immunostimulants such as Ergosan extracts (Peddie et al., 2002), levamisole (Kajita et al., 1990), muramyl dipeptide (Kodama et al., 1993), nutritional factors (Pulsford et al., 1995) and Recombinant GH (Sakai et al., 1997).

The efficacy of administration of immunostimulants in fish can presumably be reflected in the ability of these fish to resist infection. Consistent with the observed increase in several innate immune parameters, exposure of fish to *S. oligocystum* HWE significantly increased survival rates after challenge with live pathogenic *V. harveyi*. Survival rates of infected fish are usually increased after treatment with various immunostimulants (Anderson, 1992; Sakai, 1999; John et al., 2007). The brown seaweed *Undaria pinnatifida* increased the survival rate of carp against *Edwardsiella tarda* (Fujiki et al., 1994) and the disease resistance of *L. rohita* was enhanced against *A. hydrophila* when fed *A. aspera* (Rao et al., 2006). Similar results were reported after feeding large yellow croaker with glucan and challenging them with *V. harveyi* (Ai et al., 2007). Dietary supplementation of *O. sanctum* (Logambal et al., 2000) and a triherbal leaf extract through intraperitoneal injection enhanced the innate immunity and disease resistance against *A. hydrophila* in goldfish (Harikrishnan et al., 2009). Disease resistance is not only attributed to the immunostimulatory effects but also to the antimicrobial aspect of the seaweed.

For effective use of immunostimulants, timing, dosage, route of administration, and physiological conditions of fish should always be considered (Kunttu et al., 2009). Doses and method of administration of immunostimulants in the present study were similar to other studies, and the timing of the bacterial challenge was set to the time of highest level of immune function of parameters evaluated.

It can be concluded from the present results that administration of hot-water extract of *S. oligocystum* by injection or immersion effectively stimulates the non-specific humoral and cellular immune response, and protects summer flounder against *V. harveyi* challenge. However, additional studies need to be conducted to determine the immune response and disease resistance of the fish fed diets containing hot-water extracts of *S.*

oligocystum as immunostimulants. Other immune-related parameters also need to be evaluated. The economics of production and application of seaweed extracts to diets needs attention.

Acknowledgements

The first author is grateful to the United States Department of State, Institute of International Education and the Philippine-American Educational Foundation for the award of Fulbright-Philippine Agriculture Scholarship, which made this work possible. Thank you to Dan Ward and Christy Varga for their assistance with the fish.

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