

Impact of Marine Secondary Metabolites (MSM) from *Hypnea musciformis* as an Immunostimulant on Hemogram Count and *Vibrio alginolyticus* Infection in the Shrimp, *Penaeus monodon*, at Different Salinities

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Abstract

Marine secondary metabolites (MSM) from *Hypnea musciformis* positively affected immune factors in post larvae of the shrimp, *Penaeus monodon*. Shrimp were raised in one of three salinity levels and infected with one of three doses of virulent *Vibrio alginolyticus*. When challenged with 10^5 or 10^6 *V. alginolyticus* cells/ml, survival was 100% for both control shrimp and shrimp fed a commercial shrimp feed treated with the MSM extract, regardless of salinity. But, when challenged with 10^7 *V. alginolyticus* cells/ml, survival was 0 in control shrimp at all salinities, and 0, 34%, and 15% in shrimp fed treated feed and raised in 7, 17, and 27 ppt salinity, respectively.

Introduction

Antibiotic treatment of bacterial, viral, and fungal diseases can cause problems in aquaculture while post infection therapy can pose environmental dangers (Alabi et al., 1999). New drugs from seaweeds (macroalgae) have been successfully used to manage a variety of diseases (Smit, 2004). Marine secondary metabolites (MSM) from *Ulva fasciata*, incorporated into shrimp feed, have been studied as a means of treating diseases in sustainable shrimp farming (Selvin et al., 2004).

Hypnea musciformis, a red marine macroalgae, is a highly opportunistic invader, well known for its large floating blooms in coastal Maui and other regions in Asia (Smith et al., 2002). In the present study, we examined the influence of MSM from *H. musciformis* on the hemogram count (a tool for evaluating or indicating immune response) and survival after *Vibrio alginolyticus* infection at different salinities in post larvae of the shrimp, *Penaeus monodon*.

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Materials and Methods

Preparation of MSM extract. *Hypnea musciformis* were collected from the Rameswaram and Mandapam regions (079°20'E, 09°25'N) on the southeast coast of India, immediately washed in fresh sea water to remove epiphytes, sand, and other extraneous matter, and air-dried in the shade. Completely dried material was weighed and finely ground in a mechanical grinder. Secondary metabolites of the seaweed were extracted by a bulk extraction process as described by Selvin and Lipton (2004).

Preparation of feed. Commercial pelleted shrimp feed (no. 4, CP Feeds, Cochin) was used to prepare top-coated medicated feed. The crude form of *H. musciformis* (100%) was extracted using the solvent, methanol. The rotary evaporated extract, solvent-free and viscous, weighs 950 mg/ml. The standard *H. musciformis* dose required to cause immunomodulatory action in *P. monodon* is 0.950 mg per kg *P. monodon* (wet wt); in our laboratory trials, higher concentrations were found to be toxic. The standard dose was prepared by serially diluting the crude extract in double distilled water until a concentration of 0.950 mg/ml extract was obtained. One ml of the diluted extract was sprayed on 500 mg of pelleted feed using a thin-layer chromatography (TLC) sprayer, adding 4.0% gelatin as a binder. The sprayed medicated feed was dried in a hot air oven at 40±2°C.

Experimental shrimps. *Penaeus monodon* post larvae (stage 20) from the Matsyafed Shrimp Hatchery in Thirumullavaram, Kerala, were maintained in three circular 300-l high-density polymer tanks. The shrimps were acclimated to one of three salinities (7, 17, or 27 ppt) during a 2-week period. Afterward, 20 shrimp were stocked into each of eighteen 50-l glass aquaria for the feed experiment. All treatments were carried out in triplicate. Experimental shrimp were fed top-coated medicated feed while control shrimp received untreated feed, both at the daily rate of 3-4% of their body weight (Selvin and Lipton, 2004). The tanks were constantly aerated and 50% of the water was exchanged daily. Dissolved oxygen, water temperature, and pH, moni-

tored daily, ranged 4.05-5.3 mg/ml, 27.5-28.5°C, and 7.2-8.2, respectively. Average body weight was determined every ten days.

Evaluation of hemogram count. Total and differential hemogram counts were determined every 10 days. Hemolymph was withdrawn from the ventral sinus of each shrimp using a U-40 1-ml insulin syringe rinsed with the anticoagulant trisodium citrate (0.114 M trisodium citrate, 0.1 M sodium chloride, pH 7.45). The total hemogram count was estimated using a hemocytometer with four squares and expressed as the mean number of cells per ml hemolymph. The differential hemogram count indicates the percent of each type of hemocyte within the hemolymph and was estimated using a hemolymph smear. The smear was prepared on a clean glass slide and stained with Giemsa stain, often used for histopathological diagnosis of malaria and other parasites. Hemocytes were classified as hyaline, semi granular, or granular (Braak et al., 2002).

Challenge experiment. The bacterial bath challenge experiment began 30 days after the beginning of medicated feeding. *Vibrio alginolyticus* from diseased shrimp were isolated and identified in our laboratory. The bacteria was grown for 18-24 h at room temperature (30-31°C) in a nutrient agar broth supplemented with 0.5, 1.0, or 2.0% NaCl to produce the three test salinities, 7, 17, and 27 ppt, respectively. Cells were pelletized by centrifugation at 1048 x g for 10 min, suspended in sterilized water of the respective salinity, and applied to 500 ml of water in a 1-l beaker to obtain final bacterial concentrations of 10⁵, 10⁶, and 10⁷ cells/ml. Fifteen shrimp juveniles were introduced into each bacterial suspension. Challenge treatments were carried out in triplicate.

Results

Within a salinity, growth in the treated group was always higher than in the control (Fig. 1). In all three salinities, mean total hemogram count differed between the experimental and control groups (Fig. 2). The percent hyaline, semi granular, and granular hemocytes varied in no certain pattern (Fig 3). Irrespective of

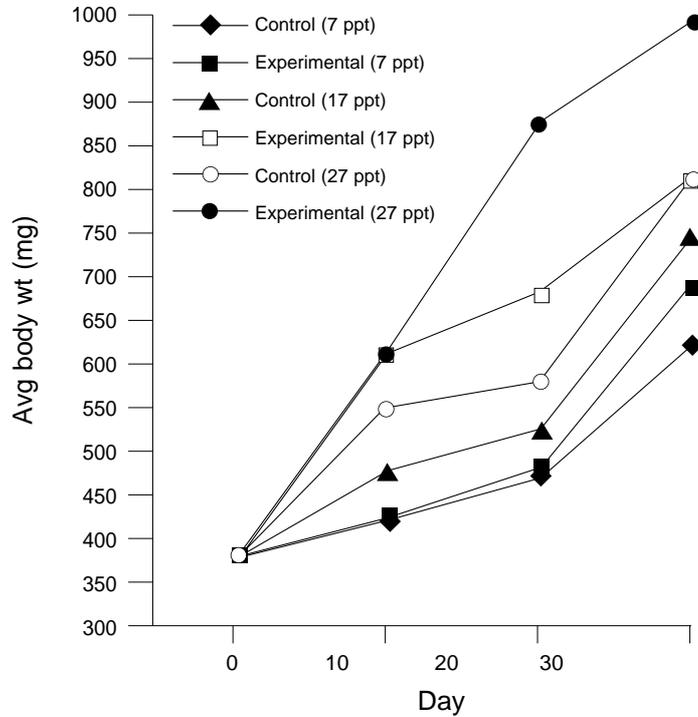


Fig. 1. Growth of *Penaeus monodon* post larvae fed a commercial shrimp feed uncoated (control) or coated with an extract of marine secondary metabolites (MSM) from *Hypnea musciformis* (experimental).

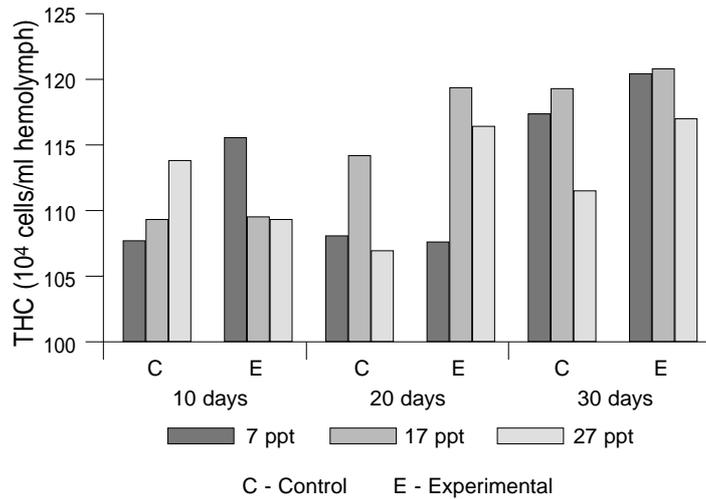


Fig. 2. Mean total hemocyte count (THC) of hemolymph of *Penaeus monodon* post larvae fed a commercial shrimp feed uncoated (control) or coated with an extract of marine secondary metabolites (MSM) from *Hypnea musciformis* (experimental).

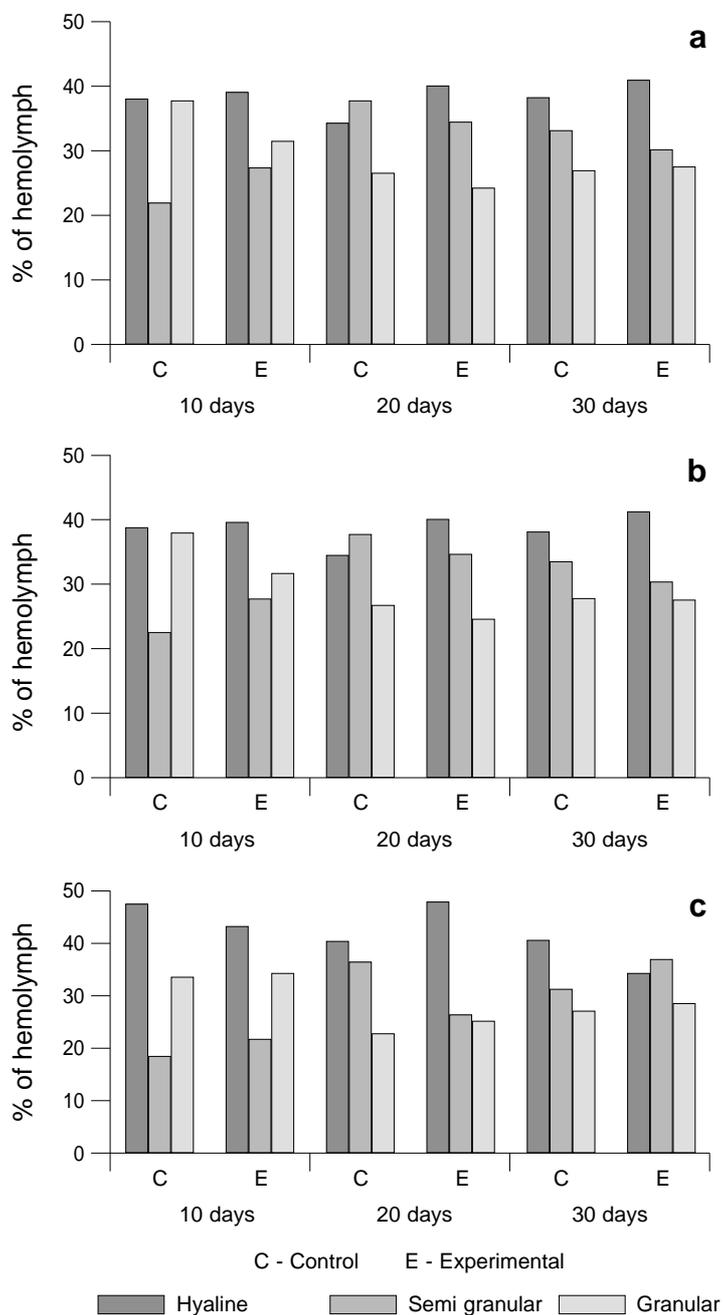


Fig. 3. Differential hemocyte count showing percentage of hyaline, semi granular, and granular hemocytes in hemolymph of *Penaeus monodon* post larvae raised at (a) 7 ppt, (b) 17 ppt, or (c) 27 ppt and fed control or MSM-treated (experimental) shrimp feed.

salinity, survival was 100% in all control and treated groups infected with 10^5 or 10^6 *V. alginolyticus* cells/ml. But when infected at 10^7 cells/ml, survival was 0 in all control groups and in the 7 ppt treated group, but 34% in 17 ppt and 15% in 27 ppt treated groups.

Discussion

MSM from macroalgae and its use in aquaculture is an emerging trend of good hope. The Rhodophyta, Phaeophyta, and Chlorophyta members of macroalgae contain secondary metabolites that enhance immunostimulant activity in shrimps (Felix et al., 2004; Selvin et al., 2004).

In the present study, there were differences in total and differential hemogram counts between the experimental and control groups in all three salinities. A similar study revealed that bioactive molecules from macroalgae enhance the proliferation of hemocytes in *P. monodon* (Huxley, 2002). In *P. paulensis*, the number of hemocytes varied 20% between shrimp raised in salinities of 34, 22, or 13 ppt (Moullac and Haffner, 2000).

Enhanced survival of treated shrimp infected by 10^7 cells/ml in 17 and 27 ppt occurred mainly after immune enhancement resulting from the MSM-incorporated feed. Feeds that incorporated seaweeds such as *U. fasciata* and *Sargassum wightii* enhanced measurable immune responses such as total hemogram count, fluctuation of the differential hemogram count agglutination titre, phagocytic rate, bacterial clearance, and proPO cascade system between treated and control groups (Felix et al., 2004; Selvin et al., 2004). It is probable that these activities are triggered by the administration of algal metabolites. In the present study, comparable immune responses in the total and differential hemogram counts were observed.

The present study indicates that incorporating MSM from *H. musciformis* in shrimp feed can act as an immunostimulant in salinities of 17 and 27 ppt. Further research is needed to understand the immunomodulatory action of *H. musciformis* incorporated feed as a successful package for sustainable shrimp farming.

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