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## Marine Secondary Metabolites (MSM) from Macro Algae Enhance Bacterial Clearance in Hemolymph of *Penaeus monodon*

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### Abstract

Marine secondary metabolites (MSM) from macro algae were incorporated into four experimental feeds for juvenile shrimp (*Penaeus monodon*) as follows: 1.0% *Hypnea musciformis* extract (diet 1); 0.1% *H. musciformis* extract (diet 2); 1.0% *H. musciformis* extract with 500 mg *Ulva fasciata* extract and 50 mg of the antibiotic levamisole (diet 3); 1.0% *H. musciformis* with 500 mg *U. fasciata* per kg body weight (diet 4). Diet 3 enhanced bacterial clearance to 99.69% in the hemolymph of shrimp challenged with *Vibrio alginolyticus* and *Vibrio fischeri*, significantly higher than clearance rates in all other treatments and the unmedicated control. Results suggest that feed containing MSM is a good alternative to application of antibiotics in controlling bacterial diseases in shrimp.

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## Introduction

Shrimp aquaculture is declining due to destructive diseases such as white spot, taura or yellow head virus, red disease syndrome, and multiple bacterial pathogens (Karunasagar and Otta, 1997; Nair, 2000). Considering economic losses and the immune system of crustaceans, immunostimulants form an important component in shrimp aquaculture by enhancing protective responses in *Penaeus monodon* (Azad et al., 2005).

Natural products of marine macro algae possess bioactive potential (Selvin and Lipton, 2004). Secondary metabolites from five sponge species inhibited the growth of eight virulent marine fish pathogens (Selva Sonia et al., 2008). Even desert plants can inhibit marine fish pathogens such as *Aeromonas hydrophila*, and *Vibrio alginolyticus* (Abutbul et al., 2005). Immune enhancement could be attributed to rapid clearance of bacteria or other extraneous particles which find entry into the hemolymph. Cells responsible for removing foreign material include circulating hemocytes and fixed phagocytes, primarily in the gills and digestive gland (Martin et al., 1993). Humoral factors such as non-self recognition proteins, prophenoloxidase, and antimicrobial peptides are produced, stored, and released from hemocytes (Holman et al., 2004). Hemocytes can adhere to a pathogen, triggering phagocytosis and thereby producing highly toxic reactive oxygen species (Song and Hsieh, 1994; Munoz et al., 2000) which help eliminate foreign particles. The humoral and cellular defense mechanisms of shrimp can be assessed by bacterial clearance assays (Sri-tunyalucksana et al., 2005).

*Hypnea musciformis* has antibacterial activity against *Bacillus subtilis* (Caccamese and Azzolina, 1979). *In vitro* studies in our laboratory indicated that *H. musciformis* contains biologically active components; a methanolic extract of *H. musciformis* exhibited multifaceted bioactivities including potent antibacterial activity against the fish and shrimp pathogens, *Vibrio alginolyticus* and *V. fischeri*, in the prawn, *P. monodon* (Jean Jose et al., 2008). A diet containing *Ulva* extract and levamisole enhanced circulating granulocytes in *P. monodon* (Huxley, 2002).

The goal of this study was to use bacterial clearance assays to examine the potential of marine secondary metabolites (MSM) from two macro algae, *H. musciformis* and *Ulva fasciata*, in clearing pathogens from the hemolymph of juvenile *P. monodon*.

## Materials and Methods

**Preparation of extracts.** Fresh macro algae were collected during the post-monsoon season. *Ulva fasciata* were collected from the coastal rocks off the southwest coast of India (Vizhinjam, 8°22'N 76°59'E) and *Hypnea musciformis* was collected from the southeast coast (Rameswaram, 9°25'N 79°20'E). The algae were washed immediately in fresh sea water to remove epiphytes, sand, and other extraneous matter, then dried in the shade, weighed, and ground finely in a mechanical grinder. Secondary metabolites were extracted according to Selvin and Lipton (2004). In this process, 500 g of finely powdered algal material was refluxed in a 5-l round-bottom flask using methanol. The extract was filtered and concentrated to recover excess solvents in another distillation system. Finally it was reduced to a thick viscous crude extract in a rotary vacuum evaporator (Buchi) at 40°C.

**Preparation of feed.** Commercial pelleted shrimp feed (grower feed no. 4; CP Feeds, Cochin) was used to prepare four experimental top-coated medicated diets (Table 1). The extracts were suspended in 100 ml of 4.0% gelatin water and sprayed on 500 mg of pelleted feed using a thin layer chromatography (TLC) sprayer. The medicated feeds were dried in a hot air oven at 40±2°C.

Table 1. Medicated top-coating of experimental diets fed to postlarvae of *Penaeus monodon* (in terms of final dosage per kg body weight of shrimp).

	<i>Experimental diet</i>			
	1	2	3	4
<i>Hypnea musciformis</i>	1.0%	0.1%	1.0%	1.0%
<i>Ulva fasciata</i>	-	-	500 mg	500 mg
Levamisole	-	-	50 mg	-

*Experimental shrimp.* One thousand healthy *P. monodon* post larvae (PL stage 6, 20-30 mg; Matsyafed Hatchery, Thirumullavaram, Kerala) were transported to the Vizhinjam laboratory and grown in captive conditions (18-20 ppt salinity, 29±2°C, constant aeration) for 75 days. They were fed pelleted feed (CP Feeds, Cochin) at a rate of 2-3% their body weight per day, divided into two feedings. When they reached 800-1000 mg, they were stocked in five 300-l fiberglass reinforced plastic tanks at 100 juveniles per tank. The tanks received constant aeration and 50% daily water exchange and were covered with mosquito netting. Shrimp were fed one of the four top-coated medicated feeds or the untreated control feed at a daily rate 4-5% body weight, divided into three feedings. Bacterial challenge experiments were initiated after 10 days of feeding.

*Bacterial clearance experiments.* Pathogenic *Vibrio alginolyticus* isolated from infected shrimps and *Vibrio fischeri* (MTCC 1738) were grown in nutrient broth and centrifuged. The pelleted cells were washed with sterile 0.85% saline and serially diluted. One set of serial dilution was used to count viable cells. After 10 days of medicated feeding, the shrimp were injected with a combination of the bacterial pathogens at the lethal dose of  $1 \times 10^5$  CFU/ml using a U-40 insulin 1-ml sterile syringe of 25 gauge (DispoVan). Shrimp were injected in the dorsal muscle tissue of the second abdominal segment and kept in separate tanks in the same conditions as above. Hemolymph samples were collected 1, 3, 6, and 9 h after injection using an anticoagulant containing 0.114 M trisodium citrate and 0.1 M sodium chloride at pH 7.4 (Cheng et al., 2002). The hemolymph was serially diluted and plated on nutrient agar to obtain counts of colony forming units (cfu). The percentage of bacterial clearance was calculated by comparing the number of viable cells to the number of inoculated cells. The bacterial challenge was repeated every ten days up to day 50.

All data were analyzed using one-way ANOVA ( $p < 0.05$  as significant level) in Microsoft Statistica Software Version 2.01.

## Results

Bacterial clearance was size-dependent (Fig. 1). The clearance rate increased as the weight of the shrimp increased. By day 40, over 90.0% of the bacteria were cleared within 1 h in all groups, including the unmedicated control. Prior to day 40, Diet 3 resulted in the highest rate of bacterial clearance and Diets 1, 3, and 4 consistently produced significantly higher bacterial clearance rates than the control, indicating the positive influence of medicated feed. Diet 2, with an inclusion of only 0.1% *H. musciformis*, did not show high bacterial clearance after day 10, suggesting the need for a higher dose.

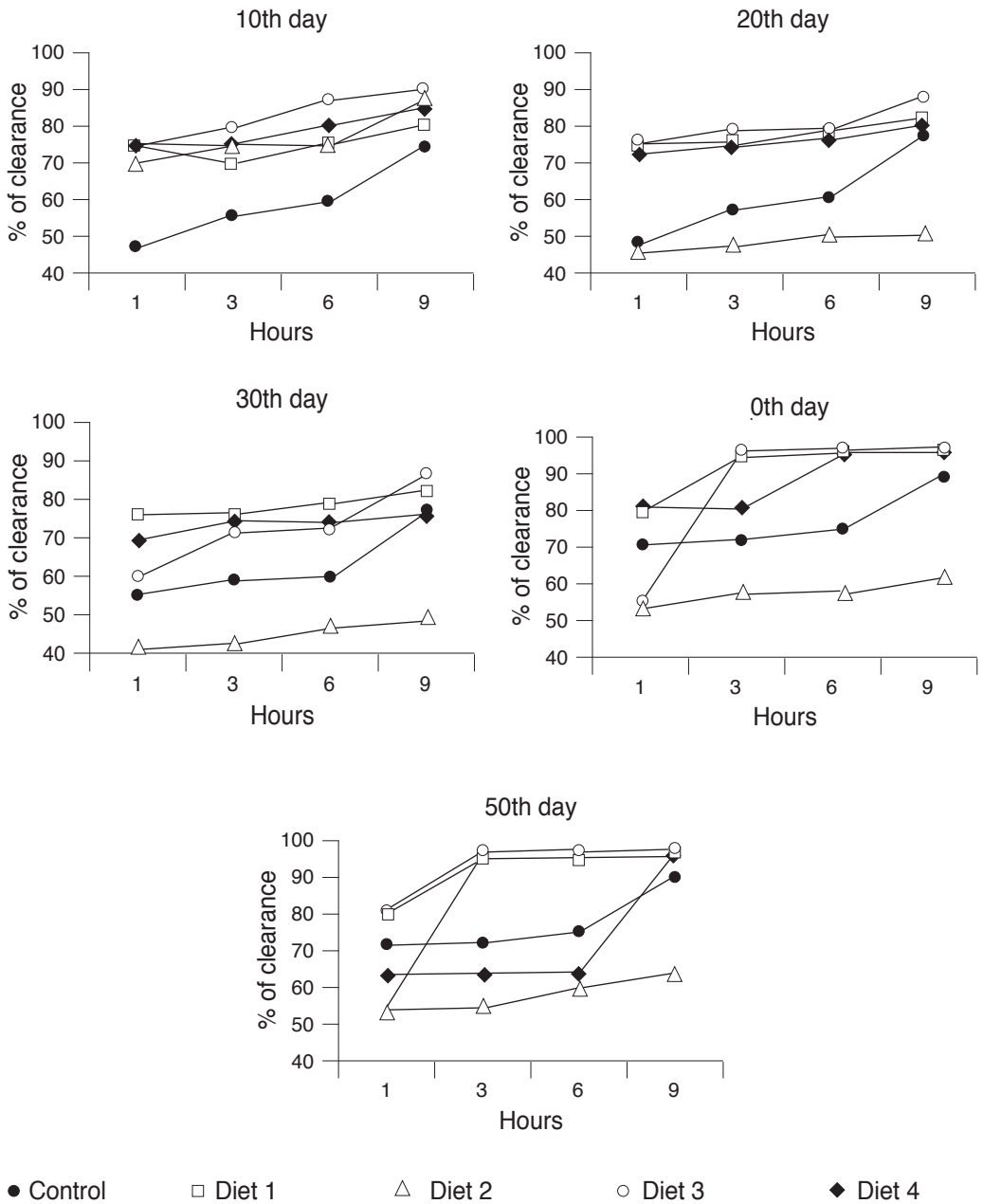


Fig. 1. Clearance of bacterial pathogens from the hemolymph of *Penaeus monodon* juveniles fed diets coated with marine secondary metabolites from *Hypnea musciformis* and *Ulva fasciata*.

### Discussion

The rapid removal of bacteria from the hemolymph as noted in the present study confirms earlier findings in other crustacean species (Merrill et al., 1979; Smith and Ratcliffe, 1980; White and Ratcliffe, 1982; van de Braak et al., 2002). The gram-positive bacteria *Bacillus cereus*, *B. subtilis*, and *Aerococcus viridans* were cleared to undetectable levels from the hemolymph of the penaeid shrimp, *Sicyonia ingentis*, within 10 min from challenge (Martin et al., 1993). The different rates at which bacteria are cleared may reflect the binding specificity of hemolymph components such as lectins (Vargas-Albores et al., 1997), anti-microbial peptides (Destoumieux et al., 2000), the prophenoloxidase (ProPO) cascade (Aspan et al., 1995), and hemocytes. The results of our combined bacterial challenge indicate that *Hypnea* is a broad spectrum vibriostatic agent.

The bacterial clearance capability of shrimp was steady in the control and experimental groups from day 40 onwards, indicating an age-dependent pattern and that shrimp hemolymph attains a high capability for removing invading pathogens. The bacteria were ultimately cleared in all treatments including the control, but the rate of clearance was improved by the medicated feed. These data support the idea that rapid clearance of live bacteria, whether by bactericidal mechanisms in the hemolymph or by physical trapping and removal to peripheral sites, contributes to disease resistance in crustaceans by limiting the spread of free pathogens to other tissues (Holman et al., 2004). The use of marine secondary metabolites from *H. musciformis* during the juvenile stage of *P. monodon* can assist in such clearance.

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