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Research Article

Efficacy of marine green alga *Ulva fasciata* extract on the management of shrimp bacterial diseases

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ABSTRACT. Secondary metabolites of the green algae, *Ulva fasciata*, were tested to determine the efficacy of controlling shrimp bacterial pathogens. Exploratory experiments indicated that an intermediate dose (1 g kg^{-1} of shrimp) of *Ulva* in the diet was highly effective at controlling bacterial pathogens of shrimp, as compared to lower (500 mg kg^{-1}) and higher (1.5 g kg^{-1}) doses. The pilot experiments evaluated the percent of relative protection afforded shrimps treated with *Ulva* diet and faced with various concentrations of bacterial pathogen. The survival of shrimps treated with *Ulva* diet was significant ($P < 0.01$). The present findings indicate that the green *U. fasciata* may be an excellent source for developing a potent medicated feed for shrimp disease management.

Keywords: *Penaeus monodon*, bacterial diseases, *Vibrio*, shell disease, proactive management, *Ulva fasciata*.

Eficacia del extracto del alga marina verde *Ulva fasciata* sobre el manejo de las enfermedades bacterianas en camarones

RESUMEN. Metabolites secundario de algas verdes *Ulva fasciata* fue probado para determinar la eficacia de controlar el camarón pathogens bacterial. Las conclusiones de experimentos exploratorios indicaron que la dosis mediana (1 g kg^{-1} de camarón) de dieta *Ulva* era sumamente eficaz en el control de pathogens bacterial de camarón cuando comparado al más abajo (500 mg kg^{-1}) y más alto (1,5 g kg^{-1}) dosis. En los experimentos pilotos, la protección de pariente de por ciento de camarones trató con la dieta *Ulva* y desafió con varias concentraciones de bacterial patógeno fueron evaluados. La supervivencia de camarones trató con la dieta *Ulva* era significativo ($P < 0,01$). Basado en las conclusiones presentes, podría ser deducido que U. verde fasciata puede ser una fuente excelente para desarrollar la comida potente medicinal para la dirección de enfermedad de camarón.

Palabras clave: *Penaeus monodon*, enfermedades bacteriales, Vibrio, enfermedad de cáscara, dirección proactiva, *Ulva fasciata*.

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INTRODUCTION

Bacterial disease outbreaks particularly vibriosis and black shell disease impose a significant constraint on the sustainable production of shrimp (Bachere *et al.*, 1995; Verschuere *et al.*, 2000; Selvin *et al.*, 2005; Manilal *et al.*, 2010b). In Asia, among the pathogenic *Vibrio* group, 11 species were reported from the shrimp culture systems (Lavilla-Pitogo, 1995). *V. alginolyticus* and *V. harveyi* poses a serious disease problem in cultured black tiger shrimp in India (Karunasagar *et al.*, 1997; Selvin & Lipton 2003a; Manilal *et al.*, 2010b). *Vibrio* species are considered to be members of the normal bacterial flora of shrimp and the culture environment (Jiravanichpaisal *et al.*, 1994; Otta *et al.*, 1999). Often acting as opportunistic pathogens or secondary invaders, they may cause total mortality of reared shrimps (Lightner, 1988; Nash *et al.*, 1992).

Although few reports evidenced the efficacy of algae-based products in shrimp health management (Furusawa *et al.*, 1991; Yamasaki *et al.*, 1997), the

role of marine natural product in shrimp disease management has been realized recently (Selvin & Lipton, 2003, 2004; Selvin *et al*. 2004b; Huang *et al.*, 2006; Manilal *et al.*, 2009). It was found that marine secondary metabolites are promising resources for the development of eco-friendly management practices for shrimp diseases (Selvin & Lipton, 2003; Selvin *et al.*, 2004, 2004b; Manilal *et al.*, 2009, 2009a). Moreover, secondary metabolites of green algae *Ulva fasciata* elicited the non-specific defense factors of shrimp against pathogenic invaders (Selvin, 2002; Selvin *et al.*, 2004b). In addition, medicated feed formulated with polysaccharides isolated from the Indian green algae, *Acrosiphonia orientalis* were found to be effective in control of White Spot Syndrome Virus (Manilal *et al.*, 2009). Recently, Huang *et al*. (2006) envisages the effect of *Sargassum fusiforme* polysaccharide extracts on vibriosis resistance and immune activity of the shrimp, *Fenneropenaeus chinensis*.

Southwest coast of India is a unique marine habitat infested with diverse variety of seaweeds. The biological activity of seaweeds from the southwest coast of India (Kollam coast) is already reported (Manilal *et al.*, 2009, 2009a, 2009b, 2010, 2010a, 2011, 2011a). In this background, the present study aims to establish the effect of algal-based medicated feed on the survival of experimentally infected shrimps.

MATERIALS AND METHODS

Collection of marine algae

The mature plants of *Ulva fasciata* was handpicked from the intertidal and subtidal habitat of the Kollam (08º54'N, 76º38'E) area located on the southwestern coast of India. The collection was performed during the period from June 2007 to August 2007 when algal biomass remains dominant. Live and healthy plants were harvested manually and washed thoroughly in running water. Cleaned plant materials were shade dried under an air jet to prevent photolysis and thermal degradation. The completely dried material was weighed and ground coarsely in a mechanical grinder (Manilal *et al.*, 2010).

Extraction of algae

For extraction, 500 g of finely powdered algal material was refluxed three times in a 5 L capacity round bottom flask in a water bath at 65ºC for about 6 h using dichloromethane: methanol (1:1) as a binary azeotropic solvent (Manilal *et al.*, 2009b). The extracts were filtered and concentrated to recover the excess solvents in another distillation system. The concentrated extract (about 100 mL) was again filtered through a Whatman Nº1 filter paper fitted with a Buchner funnel using suction pressure.

Finally, it was reduced to thick oily natured crude extract in a rotary vacuum evaporator (Yamato) at 40°C, collected in air–tight plastic vials and stored in the refrigerator for further activity studies.

Determination of median lethal dose (MLD) of algal extract

Primary exploratory (dose selecting) experiments were conducted with broad range of the test compounds. Chosen concentrations such as: 100, 1000 and 2000 mg kg^{-1} shrimp of *U. fasciata* extract were prepared in 5% EtOH in normal saline to ensure complete solubility. Ten shrimps (abw = 8.5 g) per group (triplicates) were injected intra-muscularly with 0.1 mL of appropriate dose or 5% EtOH in normal saline (control). The immediate reflexes and mortality were observed every hour for the first 6 h and every 24 h for 7 days. Based on the exploratory experiments, narrow ranges were administered to the experimental shrimp to determine the median lethal dose (LD_{50}).

Preparation of medicated diet

Top-coated medicated feed was prepared with appropriate quantity of *Ulva* extract on the surface of the feed at a rate of 3.2% of the shrimp body weight daily. To prepare the medicated feed, 30, 60 and 80 g of *Ulva* extract was incorporated in medicated diet. The shrimp were fed with a commercially formulated shrimp feed with the following proximate composition: 35.6% crude protein, 3.5% lipids, 5.9% fibre, 45.7% nitrogen-free extract and 11.21% ash. Commercial pellet shrimp grower feed No. 1 (C.P. feeds, Cochin) was used for preparing medicated feed for the experiments on leaching of *Ulva* extract in the water. The extract was dissolved in 50 mL of 4% gelatin water and sprayed on 1 kg of pellet feed using a TLC sprayer. The sprayed medicated feed was dried in a hot air oven at 40°C.

Pathogenicity and LD_{50} value of shrimp pathogens

Initially, the slant culture of shrimp pathogens (*V. fischeri*, *V. alginolyticus*, *V. harveyi* and *Aeromonas* sp.) was activated and 1 mL of 18 h culture was inoculated in 25 mL nutrient broth. This was kept overnight at 30 ± 2°C in a shaker at 180 ± 5 rpm. An 18 h shake culture was centrifuged at 4800 x g for 15 min at 40°C (Eppendorf). Cell pellets were washed twice with normal saline (NS), re-suspended and

serially diluted in NS and enumerated using a Petroff-Hausser counting chamber by plating on nutrient agar supplemented with 2% NaCl (Himedia). This was also plated on nutrient agar to get the colony forming units (cfu) values. The black tiger shrimp *Penaeus monodon* post-larvae (PL-20) obtained from the Matsyafed hatchery, Kollam and were reared in a 1000 L fiber reinforced plastic tanks provided with constant aeration and 50% daily water exchange. They were reared at optimum hydrological conditions such as salinity (35 ppt), temperature (30 ± 2°C) and pH (7.8). Healthy juveniles (average body weight = 5.4 ± 2.2 g) were segregated and maintained at 20 shrimps/tank in 200 L high-density plastic (HDP) tanks before starting the experiment. The challenged shrimps were maintained at 10 shrimps/tanks in glass aquaria. Shrimps were intramuscularly injected with 0.1 mL of bacterial inoculums using a 1 mL tuberculin syringe at ventral side between the second and third segment. Preliminary examinations revealed that challenge dose of 10^3 colony forming unit (cfu) per shrimp could not kill the injected shrimp (Manilal *et al.*, 2010b). Therefore the concentrations of 10^5 to 10^8 cfu of the appropriate single (*V. fischeri*, *V. alginolyticus*, *V. harveyi* and *Aeromonas* sp.) and combined culture (*V. alginolyticus* + *V. harveyi*) of bacteria per shrimp were used. Parallel control groups received 0.1 mL of normal saline (NS) only. Ten shrimps were used for each inoculation level. The mortality and reflexes of the shrimps were observed for every 15 min in the first hour of post-inoculation and every 1 h until the 6th h (Manilal *et al.*, 2010b). Subsequent monitoring was done every 12 h for a period of seven days (Tendencia & Dureza, 1997). The appropriate concentration of pathogens (10^5 to 10^8 cfu), which causes mortality within 24 h or within 7 days, was considered as LD_{50} whereas which causes infection or abnormalities was considered as ID_{50} (Infectivity Dose) (Manilal *et al.*, 2010b). The LD_{50} dose was calculated by the probit method, after Wardlaw (1985).

Effect of different dose of algal extracts in the survival of experimentally infected shrimps

The shrimps with an average body weight of 5.6 ± 1.8 g range were reared in the circular high density plastic (HDP) tanks at a stocking density of 60 individuals per 600 L of seawater. They were fed with *Ulva* diet (1 g kg^{-1} shrimp) in three equal installments at a rate of 3.2% of their body weight for a period of 15 days. On the 16th day, three duplicate groups each of treated shrimps (10 numbers/group) were grouped in to three and intramuscularly injected with LD_{50} (10^6 cfu/shrimp) challenge dose and higher challenge dose (10^7 to 10^8 cfu/shrimp) of single culture of bacterial pathogens such as *V. fischeri*, *V. harveyi*, *V. alginolyticus* and *Aeromonas* sp., and combined culture of pathogen (*V. harveyi*, 5 x 10^5 + *V. alginolyticus*, 5 x 10^5 = 10^6 cfu/shrimp) and transferred to the 100 L glass aquaria. They were observed for a period of 15 days for mortality and external clinical symptoms (Austin & Austin, 1989). The *Ulva* diet was continued after challenge for 15 days. Parallel control shrimps (10 numbers) received 0.1 mL normal saline only. The mortality/infectivity percentages were estimated according to Sung *et al.* (1994). The Percent Relative Protection (PRP) was determined by the following expression:

$$PRP = 1 - \left(\frac{\% \text{ of mortality (treated)}}{\% \text{ of mortality (control)}} \right) \times 100$$

To determine if significant differences existed between the treated and control shrimps, all results were analyzed using ANOVA. The significant differences were indicated at $P < 0.01$.

RESULTS

The Median Lethal Dose (MLD) of *Ulva* extract in *P. monodon* was determined to be 1120 mg kg^{-1} shrimp. During the preliminary experiment, the infection (pathogenicity range) was obtained at a higher dose of 10^8 cfu/shrimp after 5 days of inoculation with luminescent *V. fischeri* type culture. The LD_{50} values of bacterial pathogens were ranged as 2.8 x 10^7 cfu/shrimp for *V. fischeri* and 10^6 cfu/shrimp for *V. harveyi*, *V. alginolyticus* and *Aeromonas* sp. The external clinical symptoms were characterized as 'shell necrosis' and 'black spots' on the shell, necrotised chelate legs, anorexia, feeble movement and mortality.

The effect of different algal extracts in the survival of experimentally infected shrimp is presented in Table 1. The shrimp fed with the dose rate of 500-mg kg^{-1} shrimp had the lowest protection against bacterial infection. At this dose, the shrimp challenged with *V. fischeri* produced infection in 60% of challenged shrimp, whereas the shrimp challenged with *V. harveyi* and *V. alginolyticus* caused infection to the extent of 80%. The experimental group, fed with median (1 g kg^{-1} shrimp) and higher (1.5 g kg^{-1} shrimp) doses of medicated feed exhibited more or less similar level of protection. At these treatment levels, no mortality was noticed in the shrimps challenged with *V. harveyi* and *Aeromonas* sp. whereas *V. fischeri* and *V. alginolyticus* caused 20%

Table 1. Efficacy of *Ulva* diet on protection and survival of experimentally infected *Penaeus monodon.*
Tabla 1. Eficacia de *Ulva* dieta sobre la protección y la supervivencia experimentalmente se infectan *Penaeus monodon*.

Medicated feed dose rate (g kg^{-1})	Percentage of infection within 15 days*			
	V. fischeri	*V. alginolyticus*	*V. harveyi*	*Aeromonas* sp.
0.5	60	80	80	40
1.0	20	20	0	0
1.5	10	20	10	0

*100% mortality was observed in the control treatments

infection. The infected shrimps showed external clinical symptoms such as 'shell necrosis' and 'black spots' on the shell. Subsequent symptoms such as necrotised chelate legs, anorexia, feeble movement were culminated in mortality, particularly in control and lower treatments. Based on these findings, it could be envisaged that shrimp fed with 1 g kg^{-1} will provide high protection compared to other doses.

The survival rate of treated and control shrimp against various bacterial pathogens is depicted in Figures 1 to 4. The shrimps challenged with potentiated pathogen produced more infection or mortality in the treated group than those caused by the LD_{50} value of individual pathogen. In the case of *V. fischeri*, the LD_{50} did not cause mortality in the treated group while all the control shrimp died (Fig. 1). *Ulva* diet elicited significant survival (60 in terms of PRP) against the lethal dose (10^8 cfu/shrimp) of *V. fischeri*. However 20% infection was observed among the surviving shrimp of *Ulva* fed group (Table 2). The *Ulva* diet elicited complete protection (100% survival) against the infection of *V. harveyi* and *V. alginolyticus*. The inoculation of a higher dose (10^7 cfu/shrimp) of *Aeromonas* sp. caused 40% infection in the *Ulva* treated group (Table 2). It was found that the *Ulva* diet produced a higher level of protection (80% survival) against the infection caused by the combined pathogens. Invariably, the survival of treated shrimp against the bacterial infection was significant at $P < 0.01$ level (Table 2).

DISCUSSION

Based on the findings, shrimp fed with *Ulva*-based medicated feed at a dose rate of 1 g kg^{-1} could effectively control the bacterial pathogens challenged in this study. Invariably the shrimp treated and challenged subsequently with the LD_{50} of appropriate bacteria gave higher survival than the control group. In the control group, early high mortality was observed than the actual LD_{50}. It may be due to the enhanced virulence obtained by the pathogenic isolates passed through the host, prior to the 'in captivity' control experiments. The treated group challenged with higher dose (10^7 to 10^8 cfu/shrimp) of bacterial inoculum had low survival. The survival rate of *Ulva* treated shrimp was 100% against the infection caused by the shrimp bacterial pathogens. Selvin *et al.* (2004b) reported that inhibition of bacterial propagation was a possible mechanism by which *Ulva* medication provided protection from infection.

Shrimp disease management using *Ulva* diet was proven to be an effective eco-friendly management strategy for sustainable shrimp farming (Selvin, 2002; Selvin & Lipton, 2003). *Ulva* diet was found to be a potent immunomodulator and therefore it was considered as a proactive drug (Selvin *et al.*, 2004b). As per our earlier finding, 88% of viable *V. fischeri* cells were cleared-off from the haemolymph within 1 h in the *Ulva* treated group. These findings suggest the quick production of bactericidins in the haemolymph of *Ulva* treated group. The rapid bacterial clearance rate of shrimp haemocytes was stimulated by *Ulva* treatment. Therefore it was conjectured that bacteridins found in shrimp plasma might be inducibly released from haemocytes by *Ulva* medication. Recently Huang *et al*. (2006) reported the effect of seaweed *Sargassum fusiforme* polysaccharide extracts on vibriosis resistance and immune activity of the treated shrimp. Literature also evidenced the *in vivo* antiviral (WSSV) potency of seaweed-based medicated feed in *Penaeus monodon* (Manilal *et al.*, 2009). Satoh *et al*. (1987) investigated the effect of *Ulva* on susceptibility of infectious disease in red sea bream *Pagrus major* fed with 5% *Ulva pertusa* meal supplementation diet. The *Ulva* meal increased the resistance against infection caused by *Pasteurella piscicida*. A spray-dried preparation of micro-algae *Tetraselmis suecica* was reported to control a variety of fish pathogens such as *A. salmonicida*, *Staphylococcus liquifaciens*, *V. anguillarum*, *V. salmonicida* and *V. ruckeri* (Austin & Day, 1990).

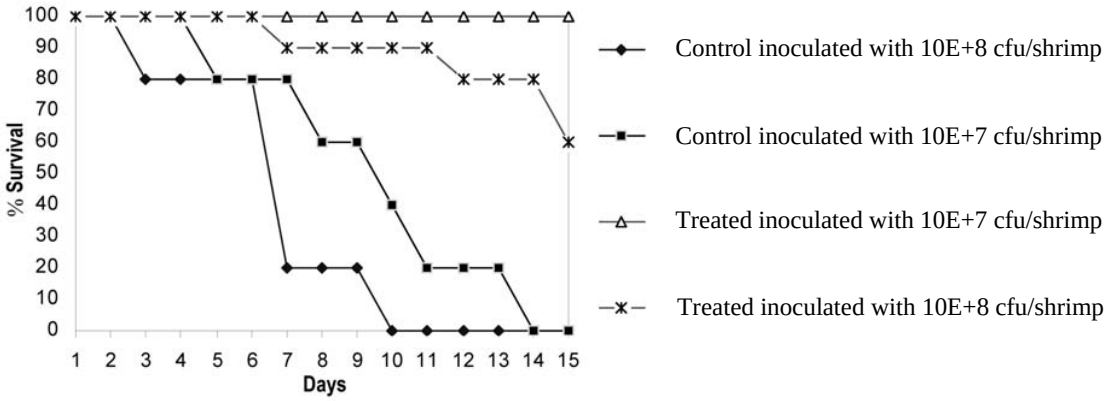

Figure 1. Survival of shrimp fed with and without *Ulva* incorporated medicated diet following challenge with *V. fischeri.*

Figura 1. Supervivencia de camarones alimentados con y sin *Ulva* incorporada como dieta medicinal para combatir el *Vibrio fischeri*.

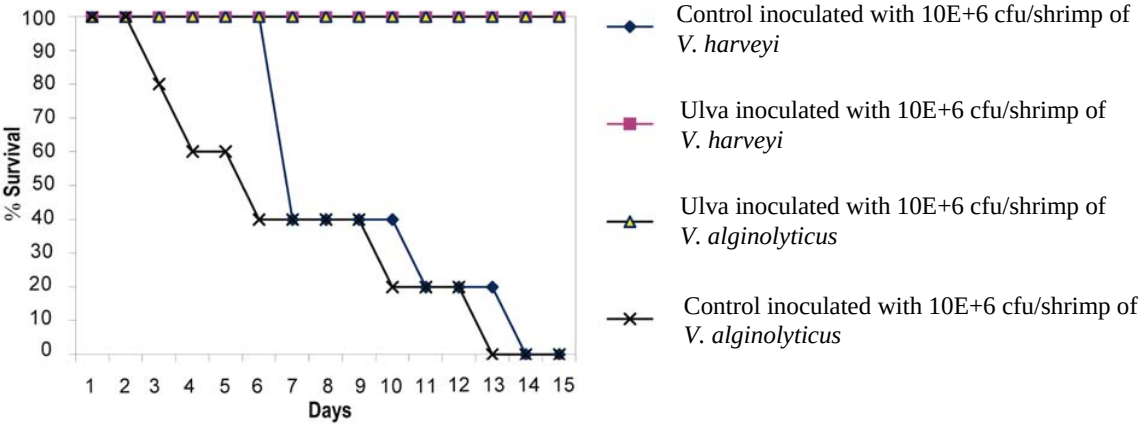

Figure 2. Survival of shrimp fed with and without *Ulva* incorporated diet following challenge with *Vibrio* sp.

Figura 2. Supervivencia de camarones alimentados con y sin *Ulva* incorporada a dietas para combatir el *Vibrio* sp.

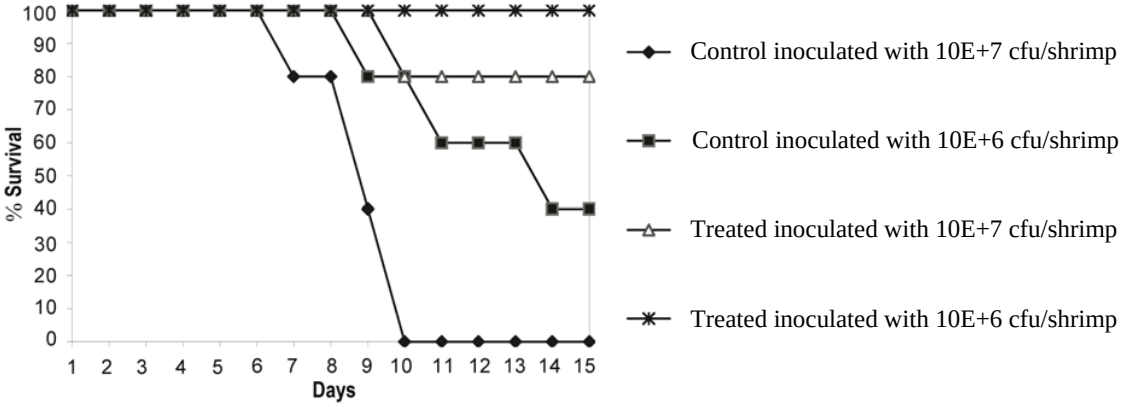

Figure 3. Survival of shrimp fed with and without *Ulva* incorporated medicated diet following challenge with *Aeromonas* sp.

Figura 3. Supervivencia de camarones alimentados con y sin *Ulva* incorporada a dietas medicinales para combatir *Aeromonas* sp.

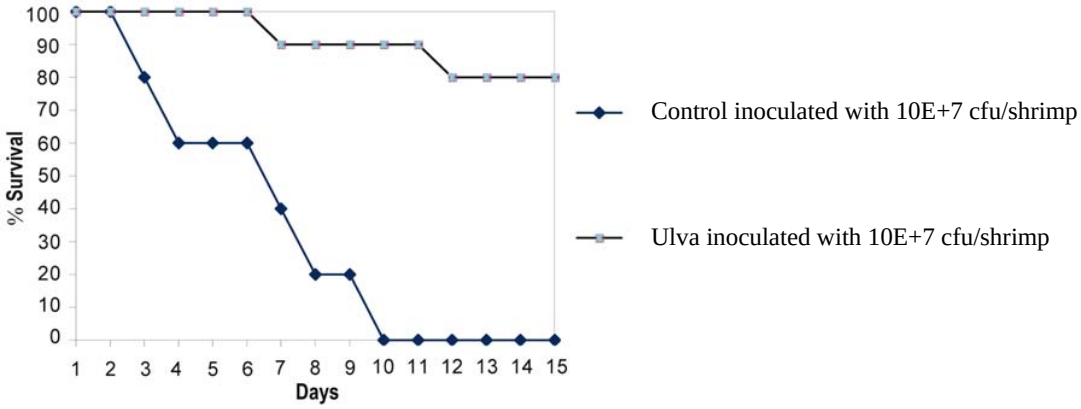

Figure 4. Survival of shrimp fed with and without *Ulva* diet following combined pathogenic challenge (*V. harveyi* and *V. alginolyticus*).

Figura 4. Supervivencia de camarones alimentados con y sin *Ulva* en dietas para combatir en forma combinada (*V. harveyi* y *V. alginolyticus*).

Table 2. Efficacy of *Ulva* diet (1000 mg kg^{-1}) on the percent relative protection (PRP) of *Penaeus monodon* against bacterial infections.

Tabla 2. Eficacia de la dieta de *Ulva* (1000 mg kg^{-1}) sobre el porcentaje de protección relativa de *Penaeus monodon* contra las infecciones bacterianas.

Bacterial pathogens	Dose (cfu/shrimp)	Cumulative Mortality within 15 days (%)* (Treated n = 20)	PRP	% shell necrosis in the treated shrimp (n = 20)
V. fischeri	10^8	40	60	20
	10^7	0	100	0
V. harveyi	10^6	0	100	0
V. alginolyticus	10^6	0	100	0
Aeromonas sp.	10^7	0	100	40
	10^6	0	100	0
Combined pathogen	10^6	20	80	0
i. *V. harveyi*,	5×10^5			
ii. *V. alginolyticus*	5×10^5			

*Based on the cumulative mortality the calculated survival rate was significant at $P < 0.01$.
100% mortality was observed in the control treatments, except *Aeromonas* sp. which was 80%

Based on the present study, therapeutic formulation (*Ulva* diet) can be easily prepared with crude extract. Therefore purification strategies and consequent synthetic analogue development process need not be undertaken. As the extract was prepared from the dried material, such source material can easily be stored for 12 months. To sustain the host-defense system and relative protection against pathogenic invaders, the *Ulva* medication can be used as a prophylactic agent for the entire culture period at low cost. Based on the present findings, it could be inferred that the secondary metabolites of *U. fasciata* form an excellent source for developing potent formulations as a package of proactive management practice for sustainable shrimp farming.

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