BIOMODULATION OF NON-SPECIFIC IMMUNE RESPONSE IN THE TIGER SHRIMP *PENAEUS MONODON* FABRICIUS WITH SPIRULINA INCORPORATED DIET

By

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PANANGAD, COCHIN

Dedicated to my

beloved parents

DECLARATION

I hereby declare that this thesis entitled "**BIOMODULATION OF NON-SPECIFIC IMMUNE RESPONSE IN THE TIGER SHRIMP *PENAEUS MONODON* FABRICIUS WITH SPIRULINA INCORPORATED DIET**" is a bonafide record of research work done by me during the course of research and that the thesis has not formed the basis for the award to me of any degree, diploma, associateship, or other similar title, of any other University or society.

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Introduction

1. INTRODUCTION

Shrimp farming is one of the fastest growing sectors in India and elsewhere in Asia. It constitutes an important source of revenue and employment in many developing countries. The black tiger shrimp, *Penaeus monodon* is the most important species used for culture in these countries.

However, incidences of both infectious and non-infectious diseases were recorded in the commercial shrimp culture systems. In recent years, several disease outbreaks have caused major problems and decimated the shrimp farming industry. Various bacterial and viral diseases have severely affected the shrimp farming (Dugger and Jory, 1999).

Attempts to control or prevent such devastating outbreaks using conventional antimicrobials and other chemotherapeutants were mostly unsuccessful and created severe environmental consequences. The uncontrolled and repeated use of antibiotics causes setbacks in successful treatment of bacterial infections due to the development of antibiotic resistant pathogens. Considering the potential threat of diseases on the one hand and the environmental issues on the other hand, disease management aspects should concentrate on environment friendly methods such as immunoprophylaxis. For this reason, disease prevention warrants priority attention and immunostimulants are one of the potential weapons shrimp farmers may use effectively against shrimp diseases.

Adaptive immunity or specific defense mechanism has been assumed to be absent in shrimps. Therefore, vaccines will not have the desired effect. The primary immune response in shrimps is non-specific cellular immunity. Haemocytes play a crucial role in this immune response because of their participation in phagocytosis, encapsulation, nodule formation, and cytotoxic mediation (Sung and Sun, 1999).

Immunostimulants are substances, which can stimulate the non-specific defense mechanism and afford protection against infection in fish, shrimp and other animals. Being natural products, they are not likely to cause any environmental hazards. Moreover, they have a broad-spectrum action against several pathogens unlike vaccines, which are specific to a particular pathogen. A number of different compounds with highly diverse chemical structures have been shown to act as immunostimulants and enhance the overall resistance of animals to a number of infectious agents simultaneously. Such compounds include bacterial cell wall fragment, beta-1, 3 glucan of yeast and mycelial fungi, peptides and a number of synthetic products (Raa, 1996). *Vibrio* bacterin, *Vibrio alginolyticus* bacterin, β-1, 3 glucan, zymosan, peptidoglycan, fucoidan, etc have been reported to be used as immunostimulants in shrimps successfully (Karunasagar and Karunasagar, 2001).

Another possible immunostimulant that has to be studied in shrimps is Spirulina. Spirulina is a helicoidal, filamentous blue-green alga or cyanobacterium found in alkaline lakes with very high pH. It is produced commercially and sold in dried powder form as a human food supplement. Spirulina contains 60-70% protein and is a rich source of vitamins, especially B_{12}, beta-carotene, minerals and gammalinolenic acid (Belay *et al.*, 1993).

In the present study, an attempt has been made to use Spirulina as an immunostimulant in the tiger shrimp, *Penaeus monodon* to enhance non-specific cellular immune response and to determine if Spirulina stimulated increased immune response against pathogen and also better colour in *P. monodon*.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Disease problem in shrimp culture industry

Disease problem in aquaculture is currently an important constraint to the growth of aquaculture. It has affected both socio-economic development and rural livelihoods in some countries (FAO, 2000). It is well known that increased disease occurrences facilitate the transfer of pathogenic organisms among and within adjoining countries. The viral disease in shrimp industry which started in one of the South-east Asian countries was quickly transmitted to many of the adjoining countries including island countries such as Sri Lanka. In Taiwan, the shrimp industry almost collapsed during the late 1980s. In India, set backs in shrimp aquaculture started from 1993 due to diseases. Thus the world production of shrimp has declined drastically due to dreadful viral and bacterial epizootics and environmental crisis. The main reason for these disease outbreaks is the higher stocking density in the shrimp farms. Because of this high stocking density, stress occurs and the delicate balance among host, pathogen and environment changes. Another reason is the introduction of brood stock from the other countries without proper quarantine measures. In shrimp, viral diseases are highly devastating followed by bacterial outbreaks. Viral outbreaks cause very high mortality reaching cent percent within 3-10 days of onset of clinical signs. According to several reports, in addition to viral diseases, majority of bacterial infections are attributable to *Vibrio* species (Adams, 1991).

Considering the potential threat of diseases on the one hand and the environmental issues on the other hand, immunoprophylaxis is the environment friendly method to control diseases.

2.2 Shrimp defense mechanism

Studies towards a better understanding of shrimp defense mechanisms constitute one approach for overcoming disease problems. Many workers have tried to study how shrimp defense factors, including possible induction mechanisms influence shrimp health and resistance to disease, but these studies have not yet led to any general conclusions (Sritunyalucksana *et al.*, 1999).

The immune system of shrimp is rather poorly understood. While it is established that the fish have well characterized specific defense mechanism, there is no definite evidence of specific defense in shrimp. However, shrimps have a wide array of non-specific factors, both cellular as well as humoral, which are involved in defense against pathogens (Soderhall and Cerenius, 1992). Non-specific cellular immunity is the primary immune response in shrimp. Haemocytes in shrimp play an essential and pivotal role in the initiation and maintenance of its non-specific immune response (Dugger and Jory, 1999). Using morphological and biochemical criteria, three types of haemocytes, viz. hyaline, semi-granular and granular, are recognized in crustaceans (Soderhall and Cerenius, 1992). Haemocytes participate in phagocytosis, encapsulation, nodule formation and cytotoxic mediation, thus plays an important role in this immune response (Sung and Sun, 1999). Foreign particles such as bacterial cells can be removed by phagocytosis or by haemolymph encapsulation, which is initiated by the proPO system (proPhenoloxidase system) (Soderhall and Cerenius, 1992). The proPO system participates in defense in a number of ways. This may lead to production of microbial compounds such as quinolones and melanin (Bachere *et al.*, 1995).

A number of haemolymph factors are associated with defense in crustaceans. These include lectins, agglutinins, precipitins, bactericidins, lysins and bacteriostatic substances (Smith and Chisholm, 1992;

Soderhall and Cerenius, 1992). Induction of such haemolymph factors in shrimps has been reported in literature.

In order to function immunologically, haemocytes must pass through a state of activation, which involves certain morphological changes. Non-activated haemocytes tend to appear smooth and spherical, while activated haemocytes are crenellated and may extrude pseudopods, which are used to capture and phagocytize pathogens. The fact that these cells produce a whole sequence of metabolic changes, which result in the production of a series of cytokines and other critical compounds, which act as internal regulators of the immune system is also very important. This activation can be initiated by several different stimuli, such as endotoxins, bacteria and also by chemical compounds, like polysaccharides. These immunostimulators are called as immunostimulants (Dugger and Jory, 1999).

2.3 Immunostimulants

Immunostimulants are substances, which elicit non-specific defense mechanisms and enhance the barrier of infections against invading pathogens. They are isolated from natural sources and then synthesized chemically. The cell wall preparations from bacteria, fungi, mushrooms and yeast are reported to contain good sources of immunostimulants (Raa *et al.*, 1992).

Perusal of literature indicates that immunostimulants have proven very successful in treating / preventing microbial diseases in cultured shellfishes. Wide varieties of both natural and synthetic analogues were developed and tested (Table1). In 1990, Robertson *et al.*, reported that the non-specific disease resistance in Atlantic salmon was enhanced by glucan preparation from *Saccharomyces cerevisiae*. Since then, several researchers have suggested the possible use of glucans against viral infections in fish and shrimps (Anderson, 1992; Raa *et al.*, 1992; Leong

Table 1: List of immunostimulants used in laboratory experiments

Species	Pathogen	Immunostimulants	Reference
P. monodon	*Vibrio*	Glucan	Rao *et al.*, 1996
P. monodon	WSSV	β 1-3 Glucan	Chang *et al.*, 1999
Marine shrimp	Virus	β 1-3 Glucan	Dugger and Jory, 1999
P. monodon *M. rosenbergi*	*Vibriosis*	Glucan Zymoson Heat killed Vibrio, LPS Trypsin	Sung *et al.*, 1998
P. monodon	*Vibriosis*	Glucan	Teunissen *et al.*, 1998
P. monodon	Microbicidal Activity	β Glucan	Sung *et al.*, 1996
P. monodon	Bacteria	*Vibrio alginolyticus* Bacterin	Adams, 1991
P. monodon	*Vibrio vulnificus*	Glucan	Sung *et al.*, 1994
P. monodon	Virus	Peptidoglucan	Itami *et al.*, 1998
Cray fish		Lipopolysaccharide & β 1-3 glucan	Lee *et al.*, 2000
P. monodon	White spot Baculo virus	Yeast β 1-3 glucan Lipopolysaccharide	Karunasagar *et al.*, 1996
Shrimp	*Vibrio*	*Vibrio* bacterin	Horne *et al.*, 1995
Shrimp	Yellow head Baculo Virus	Peptidoglucan	Boonyaratpalin *et al.*, 1995
Shrimp		Yeast glucan	Song and Hsieh, 1994

and Jo-Ann, 1993; Sakai, 1999). Controlled laboratory studies have demonstrated that immunostimulants such as vibrio bacterin, yeast glucan, peptidoglycan, schizophyllam and lipopolysaccharide, have the potential to reduce the impact of disease in shrimp (Itami *et al.*, 1989; Sung *et al.*, 1994; Song and Hseih, 1994; Itami *et al.*, 1994). Presently, several commercial immunostimulanting products are marketed for use in commercial shrimp farms.

2.4 Immunostimulants studied in fish and shellfish

According to the source materials, immunostimulants can be grouped under: Chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors and cytokines (Sakai, 1999).

2.4.1 Synthetic chemicals

2.4.1.1 Levamisole

Levamisole is an anthelminthic used for the treatment of nematode infection in man and animals. Incidental observations suggested a state of enhanced resistance to various kinds of infection upon treatment. Levamisole in mammals enhances the metabolic and phagocytic activities of neutrophils, increased the number of phagocytes and leucocytes and the level of lysozyme in serum (Symoens and Rosenthal, 1977). Coho salmon, *Oncorhynchus kisutch*, injected with levamisole mixed with modified freund's complete adjuvant (MFCA) showed increased resistance to *Aeromonas salmonicida* (Olivier *et al.*, 1985) while Siwicki (1987, 1989) reported that carp injected with levamisole showed enhanced phagocytic activity and myeloperoxidase activity in neutrophils, increased leucocyte numbers and serum lysozyme levels. Kajita *et al.* (1990) reported that rainbow trout injected with levamisole showed increased protection against *Vibrio*

anguillarum, due to the enhancement of non-specific immune responses such as phagocytic activity, chemiluminescence responses of leucocytes and NK cell activities. Immunomodulatory effects of levamisole by oral and immersion administration have also been reported. Siwicki (1989) reported that oral administration of levamisole increased the number of leucocytes, lysozyme activities in serum and stimulated NBT reduction and phagocytic index of phagocytic cells. Jency and Anderson (1993a) also showed that bathing rainbow trout for 30 minutes in levamisole solution (5 μg/ml) before a 2 minutes bath in *A. salmonicida* O-antigen bacterin elevated both the non-specific defence mechanisms (Phagocytic activity and index) and specific antibody (titers). The immunostimulating effect of levamisole has been clearly shown in fish, and this agent can be administered by various methods. The immunomodulatory effect of levamisole in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man) was determined by incorporating levamisole in dry diet by Baruah and Pani Prasad (2001). proPO activity and NBT reduction were significantly enhanced. Treated prawns challenged with a virulent strain of *Pseudomonas fluorescens* showed delayed mortalities than control.

2.4.1.2 FK-565

FK-565 (heptanoyl-y-D-glutamyl-(L)-meso-diaminopimlyl-(D)-alanine) is a peptide related to lactoyl tetrapeptide (FK-156) isolated from cultures of *Streptomyces olivaceogriseus* and has been shown to be active against microbial infection in mice (Mine *et al.*, 1983). Kitao and Yoshida (1986) found that application of FK-565 into rainbow trout increases their resistance to *A. salmonicida*, following the activation of phagocytic cells. Number of splenic antibody-producing cells and humoral antibody titers can be elevated during immunization in rainbow

trout when *Yersenia ruckeri* or *A. salmonicida* O-antigen preparations are mixed with FK-565 (Kitao *et al.*, 1987).

2.4.2 Bacterial derivatives

2.4.2.1 MDP

Muramyl dipeptide (MDP) (N-acetyl-muramyl-L-alanyl-D-isoglutamine), derived from *Mycobacterium*, elicits an immunostimulatory effect. Olivier *et al.* (1985) reported that coho salmon injected with a mixture of MDP and modified Freunds incomplete adjuvant showed 47-fold increase in resistance to *A. salmonicida*. Kodama *et al.* (1993) reported that intraperitoneal injection of rainbow trout with MDP-Lys increased the phagocytic activities, respiratory burst and migration activities of kidney leucocytes as well as resistance of the fish to *A. salmonicida* challenge.

2.4.2.2 Lipopolysaccharide (LPS)

LPS is a cell wall component of gram-negative bacteria. It can stimulate B cell proliferation, and LPS injected into red sea bream, *Pagrus major* has been demonstrated to enhance macrophage phagocytic activity (Salati *et al.*, 1987). MacArthur *et al.* (1985) reported that plaice, *Pleuronectes platessa*, injected with LPS showed increased macrophage migratory activity. *In vitro*, LPS stimulates phagocytosis and the production of superoxide anions in Atlantic salmon macrophages (Solem *et al.*, 1995). Similarly, LPS stimulates the production of macrophage activating factor in gold fish lymphocytes (Neumann *et al.*, 1995).

In shrimp, it enhances the microbicidal activity to fight microbial pathogens including viruses (Karunasagar *et al.*, 1996). Lipopolysaccharides are of different molecular weights and they

modulate the immune system. Lee *et al.* (2000) suggested that a 36-KDa LGBP plays a role in the activation of the proPO activating system in crayfish and thus seems to take part in the innate immune system of crayfish.

2.4.2.3 FCA

Freund's complete adjuvant (FCA), a mineral oil adjuvant containing killed *Mycobacterium butyricum*, enhances immune responses and increases the efficacy of vaccination in fish. Olivier *et al.* (1985) reported that coho salmon, *O. kisutch*, injected against FCA increased protection (450 times in LD50) against *A. salmonicida* challenge. This increased protection was also seen against *A. hydrophila* and *V. ordalii*. Immunologically, they demonstrated that the activation of macrophages (phagocytosis and killing) in fish injected with FCA caused increased resistance to *A. salmonicida* (Olivier *et al.*, 1986). Adams *et al.* (1988) also reported that FCA – injected rainbow trout appeared to show increased protection against furunculosis, vibriosis and red mouth disease.

2.4.2.4 Vibrio bacterin

V. anguillarum bacterin is the most successful vaccine for salmonid fish, and its efficacy is seen following administration by injection, oral dosing and immersion methods. Sakai *et al.* (1995c) reported that rainbow trout immersed in *V. anguillarum* bacterin solution showed increased protection up to 12 times in LD50 to *Streptococcus* sp. infection. The immunostimulant effect of vibrio bacterins were also reported in Kuruma shrimp *Penaeus japonicus*. Itami *et al.* (1989) reported that shrimp injected or immersed with the formalin-killed *Vibrio* bacterin experienced reduced mortalities when they were challenged with vibrio injection 30 days later. Horne *et al.* (1995) also

reported the efficacy of vibrio vaccines in black tiger shrimp. The migration of hemocytes treated with vibrio bacterin increased, compared to non-treated controls (Itami *et al.*, 1989). This fact suggested the immunostimulation of prawn and shrimp with vibrio bacterin. The component of *V. anguillarum* cells which stimulate non-specific immune responses is still unknown. However, since bacterial LPS stimulated non-specific immunity, especially macrophage activation, *V. anguillarum* LPS may act as an immunostimulant.

2.4.2.5 *A. stenohalis* and *C. butyricum*

A. stenohalis is a gram-negative aerobic organism which has been isolated from sea water. The LPS of these bacteria activates mouse macrophages and B-lymphocytes (Isogai *et al.*, 1989).

Kawahara *et al.* (1994) reported that white-spotted char, *Salvelinus leucomaenis*, injected with inactivated *A. stenohalis* showed enhanced chemiluminescent responses of kidney cells, complement activation and increased protection against *A. salmonicida* challenge.

A butyric acid bacterium, *C. butyricum*, has been used clinically to prevent disturbances of microflora in the human intestine, and oral administration of the spores of this bacterium ameliorate diarrhea, constipation and abdominal distension in man. *C. butyricum* shows immunomodulatory effects such as stimulation of macrophages and NK cells and enhances protection against Candida infection (Young *et al.*, 1987). Sakai *et al.* (1995a, b) showed enhancement of resistance to vibriosis in rainbow trout by oral administration of *C. butyricum* bacterin mediated by leucocyte activation, including phagocytosis and increases superoxide anion production.

2.4.2.6 Chitin and Chitosan

Chitin is a polysaccharide forming the principal component of crustacean and insect exoskeletons and the cell walls of certain fungi. Sakai *et al.* (1992) reported that rainbow trout injected with chitin showed stimulated macrophage activities and an increased resistance to *V. anguillarum* infection. Yellowtail injected with chitin alone, also showed increased protection against *P. piscicida* challenge, which continued until 45 days after treatment (Kawakami *et al.*, 1998).

Chitosan, de-N-acetylated chitin, also showed immunostimulatory effect. Brook trout, *Salvelinus fontinalis*, injected or immersed in chitosan solution showed increased protection against *A. salmonicida* infection (Anderson and Siwicki, 1994) as did rainbow trout administered chitosan orally (Siwicki *et al.*, 1994). Rainbow trout treated with chitosan by injection or immersion showed increase in immunological parameters in the blood such as NBT, potential killing activity, myeloperoxidase and total immunoglobulin concentration (Anderson *et al.*, 1995).

2.4.2.7 EF203

EF203 is the fermented product of chicken eggs. The oral administration of EF203 to rainbow trout stimulates the activity of leucocytes such as phagocytosis and chemilumnescence, and increases protection against *Streptococcus* infection (Yoshida *et al.*, 1993). Sakai *et al.* (1995e) reported that *R. salmoninarum* vaccinated rainbow trout receiving EF203 showed higher phagocytic activities and NBT responses in kidney leucocytes when compared to vaccinated fish without EF203 treatment or to unvaccinated fish. However, the serum agglutinating antibody titers of vaccinated fish did not show a significant increase between the EF203 and control groups although the vaccinated

fish treated with EF203 showed slightly increased survival in comparison with the other groups following *R. salmoninarum* challenge.

2.4.2.8 Glucan

The immunostimulatory effects of glucan have been well studied. The effect of several types of glucan; eg. Yeast glucan, peptido-glucan, β-1, 3, glucan (VST), have been investigated in fish. Yeast glucan is the most extensively studied of these glucans. Intraperitoneal injection of yeast glucan (β-1, 3- and β-1, 6-linked glucan) prepared from cell walls of *Saccharomyces cerevisiae* into Atlantic salmon resulted in increased resistance to *V. anguillarum*, *V. salmonicida* and *Yersenia ruckeri* (Robertsen *et al.*, 1990). Chen and Ainsworth (1992) reported that catfish injected with yeast glucan showed increased resistance to *Edwardsiella ictaluri*. However, Thompson *et al.* (1995) reported that rainbow trout injected with yeast glucan did not show enhanced protection against *V. anguillarum* infection. Yeast glucan has been applied by immersion and oral administration methods. Raa *et al.* (1992) reported that oral administration of yeast glucan to Atlantic salmon increased protection against *V. anguillarum* and *V. salmonicida*. Tiger shrimp immersed in yeast glucan solution showed enhanced protection against *V. vulnificus* infection (Sung *et al.*, 1994). Although channel catfish injected with yeast glucan showed increased protection to *E. ictaluri* (Chen and Ainsworth, 1992), oral administration did not show such an effect (Duncan and Klesius, 1996a). .

The adjuvant effect of yeast glucans has also been demonstrated. Injection of *A. salmonicida* vaccine and yeast glucan into Atlantic salmon enhanced antibody responses (Aakre *et al.*, 1994) and induced significantly increased protection against furunculosis over vaccines without yeast glucan (Rorstad *et al.*, 1993). The injection of yeast glucan alone did not show protection. Baulny *et al.* (1996) reported that oral

administration of yeast glucan to turbot immersed in *V. anguillarum* bacterin also increased protection compared with bacterin alone. As in the experiment above, yeast glucan alone did not enhance protection against *V. anguillarum* infection.

Yeast glucan enhances the lysozyme activity in Atlantic salmon, rainbow trout and turbot (Engstad *et al.*, 1992; Jorgensen *et al.*, 1993a; Thompson *et al.*, 1995; Baulny *et al.*, 1996), complement activity (Engstad *et al.*, 1992), bacterial killing activity of macrophages in rainbow trout, Atlantic salmon and catfish (Chen and Ainsworth, 1992; Jorgensen *et al.*, 1993a, b; Jorgensen and Robertsen, 1995) and the production of superoxide by macrophages or hemocytes in rainbow trout, tiger shrimp, catfish and Atlantic salmon (Jorgensen *et al.*, 1993a; Song and Hsieh, 1994; Yoshida *et al.*, 1995; Baulny *et al.*, 1996).

β-1, 3 glucan (VST) derived from *Schizophyllum commune* has also been evaluated as a protection against disease. Coho salmon treated with VST by injection or oral routes showed enhanced protection against *A. salmonicida* (Nikl *et al.*, 1991, 1993). However, Chinook salmon immersed in a solution of this glucan did not show protection (Nikl *et al.*, 1993).

Peptidoglucan prepared from *Brevibacterium lactofermentum* increased phagocytosis in yellowtail and resistance to *Enterococcus seriola* infection (Itami *et al.*, 1996). The efficiency of peptidoglucan was demonstrated against yellow-head baculovirus infection in black tiger shrimp (Boonyaratpalin *et al.*, 1995). The efficiencies of glucan supplied by Sigma (Jeney and Anderson, 1993b), Lentinan, Schizophyllum and Scleoglucan (Yano *et al.*, 1991; Matsuyama *et al.*, 1992; Itami *et al.*, 1994) have also been demonstrated in carp, yellowtail and prawn.

In the treated shrimps, the disease resistance could be correlated with enhanced phenoloxidase activity and intrahemocytic production of super oxide anion (Sung *et al.*, 1996).

Studies undertaken to evaluate the potency of glucan as an immunostimulant in crustaceans have proved successful. The dietary incorporation of beta-1, 3-glucan from *Schizophyllum commune* enhanced the resistance of post-larvae, juvenile and adult *Penaeus monodon* to white spot syndrome virus (WSSV) (Chang *et al.*, 1999, 2000) and against *Vibrio* (Rao *et al.*, 1996). The phenoloxidase (PO) activity of hemocyte lysate supernatant (HLS) from both tiger shrimp *Penaeus monod*on and giant freshwater prawn *Macrobrachium rosenbergii* was enhanced significantly on treatment with β-1, 3 and β-1, 6 linked glucan at the rate of 1 mg/ml (Sung *et al.*, 1998).

2.4.3 Animal and plant components

2.4.3.1 Animal extracts

Eel injected with Ete (an extract from the marine tunicate, *Ecteinascida turbinata*) showed enhanced phagocytosis and increased survival following *A. hydrophila* challenge (Davis and Hayasaka, 1984). However, Stanley *et al.* (1995) demonstrated that the survival after infection with *E. ictaluri* decreased in channel catfish injected with Ete, although immune enhancement was observed. Rainbow trout injected with Hde (a glycoprotein fraction of water extract from abalone, *Haliotis discuss hannai*) also showed enhanced phagocytosis and NK cell activities. Increased survival against *V. anguillarum* infection was also observed (Sakai *et al.*, 1991).

2.4.3.2 Glycyrrhizin

Jang *et al.* (1995) reported that *in vitro* treatment with glycyrrhizin enhanced the respiratory burst activity of macrophages and the proliferative responses of lymphocytes from rainbow trout.

2.4.3.3 Other immunostimulants

Rainbow trout treated with soybean protein showed increased leucocyte activities such as phagocytosis, bacterial killing and the production of superoxide anion (Rumsey *et al.*, 1994). The bath administration of Quil A saponin with *Y. ruckeri* vaccine enhanced the *in vitro* bactericidal activities in rainbow trout (Grayson *et al.*, 1987). Ninomiya *et al.* (1995) reported that oral administration of Quil A saponin increased leucocyte migration in yellowtail. The immunostimulant effect of PS-K (protein – bound polysaccharide) was also reported in tilapia, *Oreochromis niloticus* by Park and Jeong (1996).

Considering the inadequate and costly commercial products, marine natural products may form excellent sources for developing potent cost-effective immunostimulants. Earlier reports clearly indicated that many of such products exhibit potent immunostimulatory effect in the *in vitro* and *in vivo* assays. In seaweeds, sulfated polysaccharides have been found to be immunostimulatory (Hanazawa *et al.*, 1982). Successful protection against microbial pathogens was observed in the animals administered with the extracts as prophylactics (Rama, 1996).

2.4.4 Diet Components

High levels of dietary vitamin C are reported to increase resistance to *Edwardsiella tarda* and *E. ictaluri* infection in channel catfish (Durve and Lovell, 1982; Liu *et al.*, 1989) to *V. anguillarum* and *Icthyopthirius multifilis* in rainbow trout (Navarre and Halver, 1989; Wahli *et al.*, 1995) and to *A. salmonicida* and *V. salmonicida* in Atlantic salmon

(Erdal *et al.*, 1991; Hardie *et al.*, 1991). Hardie *et al.* (1991) showed that treatment with high doses of vitamin C increased complement activities in Atlantic salmon. However, Liu *et al.* (1989) did not observe such effects in catfish. On the other hand, the activation of macrophages was reported by Thompson *et al.* (1993) in Atlantic salmon and by Roberts *et al.* (1995) in turbot. Hardie *et al.* (1991) reported that treatment of fish with high doses of vitamin C stimulated macrophage-activating factors, followed by lymphocyte proliferation. These results show that fishes fed with high doses (more than 1000 mg/kg) of vitamin C have protective immune responses.

Blazer and Wolke (1984) reported that specific and cell-mediated immunity and macrophage phagocytosis were all compromised in rainbow trout fed vitamin E-depleted diets. Furthermore, Hardie *et al.* (1990) reported that Atlantic salmon fed vitamin E-depleted diets had significantly increased mortality rates following *A. salmonicida* infection compared to fish on the commercial diet. However, only complement activity was compromised in these vitamin E-depleted fish. Wise *et al.* (1993a, b) showed that catfish fed high doses of vitamin E had increased phagocytic indices and superoxide anion production by leucocytes.

Other dietary components such as lipids, other vitamins, trace elements, etc. can affect immune responses in fish. The importance of diet in fish immune responses was reviewed by Landolt (1989) and Blazer (1992).

2.4.5 Hormones, cytokines and others

2.4.5.1 Hormones

In fish, exogenous growth hormone (GH) has mitogenic activity on lymphocytes and activates NK cells (Kajita *et al.*, 1992; Sakai *et al.*,

1996b). In addition, Sakai *et al.* (1995d, 1996a) reported that exogenous GH given to rainbow trout increased the production of superoxide anion in leucocytes. Sakai *et al.* (1996c) reported that injection of rainbow trout with GH increased serum hemolytic activities. Furthermore, the injection of GH increased protection against *V. anguillarum* in rainbow trout (Sakai *et al.*, 1997).

Prolactin (PRL) also has immunostimulatory activities. Sakai *et al.* (1996 b) reported that the addition of homogenous PRL to chum salmon, *Oncorhynchus keta* induced lymphocyte mitogenic responses and PRL increased the production of superoxide anion by leucocytes in rainbow trout (Sakai *et al.*, 1996a).

2.4.5.2 Cytokines

Cytokines are polypeptides or glycoproteins which act as modulators in the immune system. The existence of several cytokines has been reported in fish (Secombes *et al.*, 1996).

2.4.5.3 Lactoferrin

Lactoferrin, which consists of a single peptide chain with a molecular weight of about 87 Kd and possessing two Fe-binding sites per molecule, is widely distributed in the physiological fluids of mammals. It has also been found to regulate hydroxyl radical production by macrophages, granulocytes and neutrophil leucocytes (Amberuso and Johnston, 1981). Sakai *et al.* (1993) reported that rainbow trout orally treated with bovine lactoferrin showed enhanced phagocytic activities and the production of superoxide anion by macrophages. They also observed that the fish has high resistance to *V. anguillarum* infection but there was only marginal increase in resistance to *Streptococcus* sp. Lactoferrin can activate macrophages *in vitro* (Sakai *et al.*, 1995b).

2.4.6 Spirulina

Spirulina is a single celled, spiral-shaped blue-green microalgae. Spirulina provides vitamins, many minerals, essential amino acids, carbohydrates and enzymes. A highly digestible food, spirulina contains at least 60% vegetable protein, very high carotenoid pigment content of about 4 mg/gm (Venkataraman, 1983) and Chlorophyll-a about 1.7% of the organic cell weight (Richmond, 1988). Although the amino acid content of Spirulina is generally well balanced, it is low in sulphur containing amino acids and tryptophan (Venkataraman, 1983). High content of fatty acids were also reported in Spirulina. The most important fatty acid components are linoleic acid and linolenic acid, which in Spirulina were 1.24% and 1.04% of the dry matter respectively (Richmond, 1988).

The cynobacterium Spirulina assumes significance since the cell wall of Spirulina is made up of mucoproteins (Venkataraman, 1983) and there are no associated toxic products (Richmond, 1988). Blue green algae in general and Spirulina in particular are unique in that they are highly digestible and thus do not require special processing (Richmonds, 1988).

Spirulina is being widely promoted as a nutritional supplement. It is also believed to enhance the immune system of organisms. Investigations are underway to determine the immunostimulating potential of Spirulina. Animals experimentally fed Spirulina were afforded significant protection from the effects of high doses of dioxin, gentamicin, cisplatin and organic mercury (Qureshi and Ali, 1996). Since it has a prokaryotic type of cell structure, the cell wall components affect the immune system of man and animals. Thin cell wall is made up of polysaccharides and muramyl peptides. Spiurlina polysaccharides

have both antioxidant and immune system stimulating properties (Hayashi *et al.*, 1994).

Many *in vitro* and *in vivo* studies have demonstrated that Spirulina can stimulate the immune system functions, especially those mediated by macrophages, which are the primary phagocytes. For instance, Hayashi *et al.* (1994) showed that Spirulina enhances the immune response, particularly the primary response, by stimulating macrophage functions, phagocytosis and interleukin-1 production in mice. In their studies with *Spirulina platensis* extracts, Qureshi and Ali (1996) also observed the enhancement of macrophage function. Chicken macrophages exposed to a water-soluble spirulina extract showed enhanced phagocytic activity *in vitro* (Qureshi, *et al.*, 1995).

Spirulina has also been used to enhance immune response in fish. Duncan and Klesius (1996b) reported that use of Spirulina in feed of channel catfish resulted in enhanced antibody responses to KHL, a thymus dependent antigen, but not to *E. ictaluri*, a thymus independent antigen. James *et al.* (1992) showed that *Spirulina fusiformis* cannot serve as a sole protein source in the diet of *M. rosenbergii* but can be effectively used as a supplementary protein. Good pigmentation was found after feeding trial, which seems to bear some relation with the high quality of carotene in the Spirulina. But there seems to be no reports on immunostimulating property or growth promoting activity of Spirulina in Penaeids.

2.5 Timing of immunostimulants administration

The effect of timing the administration on immunostimulant function is a very important issue. In the case of antibiotics usually, the most effective timing depends on the occurrence of disease, and they cannot often be used prophylactically due to risk of fostering the development of drug-resistant bacteria. Anderson (1992) proposed that

immunostimulants should be applied before the outbreak of disease to reduce disease-related losses.

Immunostimulants can promote recovery from immunosuppression states caused by stress. Kitao and Yoshida (1986) reported that rainbow trout injected with cyclophosphamide or hydrocortisone showed suppressed phagocytic activity of peritoneal and kidney leucocytes, and this suppression of the phagocytosis was reversed by injection of FK-565. Boonyaratpalin *et al.* (1995) also showed that peptidoglycan-fed black tiger shrimp exhibited a higher tolerance to dissolved oxygen, salinity, and stress than those fed the control diet.

The effective timing of administration of immunostimulants such as Ete is very difficult. Ete exerted a protective effect in eels injected intraperitoneally two days after challenge with *A. hydrophila.* However, the protection was not seen when Ete was administred intraperitoneally two days before or concurrently with the bacteria (Davis and Hayasaka, 1984).

2.6 Route of administration

Many authors reported that the injection of immunostimulants enhances the function of leucocytes and protection against pathogens. However, this method is labour intensive, relatively time-consuming and becomes impractical when fishes weigh less than 15 g. Thus, other methods such as oral administration or immersion should be used. Oral administration of immunostimulants has already been reported for glucans, EF203, lactoferrin, levamisole and chitosan. This method is non-stressful and allows mass administration regardless of fish size. Oral administration of these immunostimulants results in enhancement of leucocyte function and protection against infectious diseases such as furunculosis, vibriosis, streptococcosis. Anderson *et al.* (1995)

demonstrated that rainbow trout immersed with glucan or chitosan showed increased protection against *A. salmonicida* after treatment for three days. However, this effect was transient and was not present after fourteen days. Adjuvant effects of immunostimulants administered by immersion have also been reported by several authors. Jeny and Anderson (1993a) reported that rainbow trout bathed in *A. salmonicida* O-antigen in combination with immunostimulants (levamisole, quarternary ammonium compound, polypeptide of fish extract) for 30 minutes enhanced phagocytosis by leucocytes and antibody titers against *A. salmonicida*, and showed adjuvant effects with vaccination. Nikl *et al.* (1993) compared adjuvant effect of glucan against *A. salmonicida* vaccine oral delivery (7 days administration) and immersion (15min). No adjuvant effects were seen with the immersion treatment, although the fish administered glucan orally showed enhanced vaccine effects. The efficacies of immersion administration of immunostimulants has been demonstrated by several authors. However, the dilution and the levels of efficacy require more complete investigation.

2.7 Doses

Immunostimulants increase the immune responses and enhance protection against pathogens, which raises the question of dose-dependency. Kajita *et al.* (1990) showed that the chemiluminescent effect of phagocytic cells in rainbow trout were increased by injection of levamisole at 0.1 and 0.5 mg / kg. However, they also reported that the injection of 5 mg / kg of levamisole did not produce this immunostimulant effect. Kitao *et al.* (1987) reported that high doses (10 μg / kg) of FK 565 did not increase the numbers of plaque-forming cells (PFC) against *Y. ruckeri*, although the optimum dose (5 μg / kg) increased PFC. The effects of immunostimulants are, therefore, not

directly dose-dependant, and high dose may not enhance but may inhibit the immune response.

2.8 The effect of long-term administration

Oral administration is the most practical method for delivery of immunostimulants. However, the effects of long-term oral administration are still unclear. Yoshida *et al.* (1995) demonstrated that the number of NBT-positive cells in African catfish increased following oral administration of glucan or oligosaccharide over 30 days, but not over 45 days. Although the reason for this decrease in immune responses in fish by long-term oral administration of immunostimulants is still unknown, negative feedback systems against immunostimulation may function in fish, and the immune responses may revert to a previous state. Thus, the effective administration period should be investigated for each immunostimulant (Yoshida *et al.*, 1995).

2.9 The additional effects of immunostimulants

Several authors have reported relationship between immunostimulation and growth promoting activity. Boonyaratpalin *et al.* (1995) reported that black tiger shrimp fed with peptidoglycan-supplemented feed showed better growth and feed conversion rates than those fed a normal diet. This effect was observed with 0.01% peptidoglycan supplementation, but not with the highest-level administration (0.1%). Sung *et al.* (1994) also showed that black tiger shrimp grew faster with glucan immersion at the 0.5, 1, and 2 mg/ml than at 0.25 mg/ml or in control solution. Sakai *et al.* (1995d, 1996a,b) showed that GH also functions as an immunostimulant and enhances macrophage activities of fish. Rainbow trout injected with GH exhibited increased resistance against *V. anguillarum* challenge. Thus, there may be a close correlation between growth and immunostimulation.

Materials and Methods

3. MATERIALS AND METHODS

The present study was done to investigate the use of Spirulina in feed as immunostimulant to enhance nonspecific immune response in tiger shrimp, *Penaeus monodon*. The experiment was conducted at the College of Fisheries, Panangad, Cochin.

3.1 Experimental animal:

Penaeus monodon post larvae were obtained from the Aquaplaza hatchery, Cherai, Cochin. These post larvae were transported from the hatchery to College in airtight oxygen packed polythene bag. These post larvae were acclimatized at 20 ppt. salinity in a flat bottom fiberglass tank of 1.2 tons. Gentle aeration was provided using air diffusion stones. The post larvae were fed twice daily with granulated artificial feed having clam meat as the main source of protein. Left over feed and waste were removed daily by siphoning and 50% of water was renewed every day. Twigs and PVC pipes were provided at the bottom of the tank in order to reduce cannibalism. The post larvae of average weight 100 mg and average length 1.9 cm were grown to juveniles of 1.7 gm and 6.2 cm in 2 months. These juveniles were used for the present study.

3.2 Experimental set up:

The diet evaluation experiment with *P. monodon* juveniles was conducted in the wet lab of Department of Aquaculture, College of Fisheries, Panangad. Flat bottom circular fiberglass tanks having the following specifications were used for rearing the test animals.

Capacity of tank	:	83 lit.
Diameter	:	55 cm
Height	:	35 cm

Thickness of wall	:	1 mm
Rim width	:	3 cm
Colour	:	Aquamarine

Clear brackishwater of 20 ppt salinity filtered through a close meshed nylon blotting silk was used for filling the tanks up to a height of 25 cm.

3.3 Immunostimulant:

Spirulina capsules used as an immunostimulant for this study were produced by Parry Nutraceuticals Ltd. and purchased locally.

3.4 Experimental feed:

3.4.1 Feed Ingredients

Five experimental diets (T_1-T_5) were prepared for *P. monodon* juveniles at same level of protein, 40% and different percentage of Spirulina viz. 0%, 10%, 20%, 30% and 40%. In each feed, clam meat was used for replacement to keep the percentage of crude protein at 40%. The percentage compositions of the diets are given in Table 2. Control diet (T_1) was prepared by using defattened clam meat, rice bran and groundnut oil cake autoclaved for 20 min at atmospheric temperature. Then cod liver oil, vitamin-mineral mix and binder "bindex" (Matrix Vet Pharma (P) Ltd) was used for all feeds. Defattened clam meat powder was obtained by employing the following procedure. Clam meat was sun dried, pulverized and sieved to obtain a fine powder. The powdered clam meat was subjected to solvent extraction using petroleum ether (60-80°C) in a solvent extraction apparatus till the crude fat was removed. Vitamins and minerals were supplemented through Supplevit-M (Sarabhai Chemicals, Mumbai). Cod liver oil (Universal Medicare Ltd.) was used as the lipid source.

Good quality rice bran and groundnut oil cake were purchased. Polythene bags were used to store the powdered ingredients after sieving through 250 μ mesh.

3.4.2 Diet formulation

The test diets were prepared by accurately weighing the respective ingredients in an electronic balance. Table 2 gives the proportion of the ingredients used in the preparation of the formulated diet. The defattened clam meat, rice bran and groundnut oil cake were mixed well. The dry mixture was made into soft dough by adding potable water at the rate of 125 ml per 100 g of feed and mixed well. The dough was transferred into a glass beaker and steam cooked for 30 minutes in an autoclave at ambient pressure. The cooked dough was cooled well under fan. Supplevit-M, cod liver oil, Spirulina and bindex were added to this and mixed well. Then it was extruded through a pellatizer and spread in an enamel tray and dried in an oven at 65°C for 7 hours, for reducing moisture to less than 10%. After drying, the pellets were broken into small pieces and stored at a temperature of less than 4°C in airtight plastic containers until it was fed to the experimental animals.

Table No. 2 Ingredient composition of experimental feeds

Feed No.	% Spirulina	% Clam meat	% Rice bran	Groundnut oil cake (GOC)	Cod liver oil	Vit Mix
1	0	40	38.4	19.6	1	1
2	10	30	38.4	19.6	1	1
3	20	20	38.4	19.6	1	1
4	30	10	38.4	19.6	1	1
5	40	0	38.4	19.6	1	1

3.4.3 Proximate Analysis of feed:

Proximate analysis of feed was done for evaluating the nutritional status of the feed. For each feed, three replications of analysis were done and their mean was taken as the result. The methods used for analysis are shown below:

Moisture content of the feed %`	:	Drying the sample at 105°C till a constant weight was arrived.
Crude fat %	:	Solvent extraction using petroleum ether (BP 60-80°C) in a soxhlet extraction apparatus for 6 hrs.
Crude protein %	:	Microkjeldhal's method (AOAC, 1984)
Ash content %	:	Burning the sample at 550°C ±10°C for 6 hrs. in a muffle furnace.
Crude fibre %	:	Pearson method (1976)
Carbohydrate %	:	100 – (% protein + % fat + % ash + crude fibre %) by difference method (Hasting, 1976)

3.5 Experimental design and procedure

Flat bottom circular fibreglass tanks were used for the experiment. 200 numbers of healthy uniform sized juvenile shrimps (average weight –1.7 gm) were selected from a population of 500 numbers and 10 numbers each were randomly distributed in twenty experimental tanks after taking their initial weights. The experiment was conducted in a completely randomized design with five treatments and four replications each. Before the commencement of feeding with

the experimental diet, the juveniles were conditioned with the control diet for 5 days.

Each treatment group of animals was fed *ad libitum* with corresponding diets once a day at the rate of 6% of biomass for first 15 days. The leftover feed was collected daily and dried at 60°C for estimating the quantity of feed consumed. For the next 20 days the rate of feeding was 4% of the biomass.

Every day before feeding, the sides of the tank were cleaned, waste was siphoned out and 50% of water exchanged by adding freshly prepared water of 20 ppt salinity.

After the first fortnight, sampling was done to determine the weight gain by the juveniles. Weighing was done individually and average weight was computed.

Duration of the feeding study was 5 weeks. At the end of the experiment, the average weight and length of the shrimps in each treatment and replication were recorded.

3.6 Water quality parameters monitored

Temperature and salinity of the water used for the experiment were checked daily while dissolved oxygen and pH were monitored at weekly intervals. During the experiment, water quality parameters such as temperature, salinity and dissolved oxygen were checked by the following methods.

Temperature	:	By using Mercury thermometer of 0.10°C
Salinity	:	By using refractometer
pH	:	By using universal indicator (Qualigens) solution
Dissolved Oxygen	:	Winkler's method (Strickland and Parsons, 1972)

3.7 Chemicals and equipments

All the chemicals and reagents were obtained from Loba Chemie Pvt. Ltd. Disposable pyrogen-free Eppendorf tubes (Tarsons) and syringes (Dispo van) were used to collect shrimp haemolymph and sterile microwell plates (Tarsons) were used for agglutinin assay. Agar plates prepared with Himedia agar were used for antibacterial assay. All glasswares were sterilized by heating at 110°C for 1h.

3.8 Preparation of haemolymph samples:

Haemolymph samples of 100-200 µl were taken from the ventral-sinus cavity of individual living shrimp. Haemolymph samples from 6 animals were taken from each tank and pooled. Haemolymph was drawn into a syringe containing anticoagulant (Alsevier's solution: 2.05g Glucose, 0.8 g trisodium citrate, 0.055 g citric acid, 0.42 g sodium chloride in 100 ml triple distilled water).

3.9 Bacteria

For the study of antibacterial factors and for bacterial challenge, a pathogenic strain of bacteria *Vibrio parahaemolyticus* was obtained from the Pathology Department, C.M.F.R.I., Kochi. The isolate was grown in nutrient broth with 1% saline.

3.10 Preparation of Human erythrocytes

Human blood (group O) samples were obtained by venous puncture, and then collected in sterile Alsevier's solution. Before use, packed red blood cells (RBC) were prepared by centrifugation of whole blood at 5000 RPM for 10 min. The packed RBC were washed twice by centrifugation with sterile Tris-buffer saline (TBS; 50 mM Tris (Hydroxymethyl aminomethane), 100 mM NaCl, pH 7.3) and then resuspended at 1.5% (v/v) in TBS.

3.11 Evaluation indices

The parameters evaluated were percentage weight gain, specific growth rate (SGR), food conversion efficiency, food consumption rate and colour. For studies on immune response, parameters evaluated were antibacterial assay, haemagglutinin assay, phenoloxidase assay, phagocytosis and challenge study.

3.11.1 Percentage weight gain

Percentage growth of test animals were calculated by using the following formula

$$\text{Percentage weight gain} = \frac{\text{Average final weight} - \text{average initial weight}}{\text{Average initial weight}} \times 100$$

3.11.2 Specific Growth Rate

In the present study, growth performance was also measured in terms of specific growth rate (SGR) since it is a more refined and an improved growth index than absolute growth or percentage growth.

$$SGR = \frac{\ln W_2 - \ln W_1}{T_2 - T_1}$$

Where, W_1 = Weight at time T_1

W_2 = Weight at time T_2

$T_2 - T_1$ = Duration of experiment in days.

3.11.3 Food Conversion Ratio (FCR)

FCR is the ratio between the weight of food consumed and the weight gain of the animal, which often serves as a measure of efficiency of the diet.

$$FCR = \frac{\text{Average weight of food consumed in dry weight}}{\text{Average live or wet weight gain}}$$

3.11.4 Food Conversion Efficiency (FCE)

FCE is the wet weight gain of animal per unit of food consumed and this was calculated using the formula.

$$FCE = \frac{\text{Wet weight gain of shrimp (g) during the sampling period}}{\text{Dry weight of food consumed (g) during the sampling period}}$$

3.11.5 Survival rate at the end of feeding experiment

Mortality of the shrimps during the experiment was noted from each tank and percentage of survival at the end of feeding experiment was calculated as follows

$$\text{Percentage survival rate} = \frac{\text{Number of shrimps survived}}{\text{Number of shrimps stocked}} \times 100$$

3.11.6 Phenoloxidase assay

Phenoloxidase activity in haemolymph samples was determined by using L-dihydroxyphenylalanine (L-DOPA) as a substrate (Soderhall, 1983). TBS (200µl) was added to experimental cuvette containing 200µl haemolymph samples. Then, 400µl L-DOPA solution (1.6 mg/ml in TBS) was added, followed by immediate mixing. Next, 1.2 ml TBS was added as diluent and enzyme activity was determined by measuring the absorbance of dopachrome at 490 nm against a blank containing 1.6 ml TBS and 400µl L-DOPA. The absorbance value at 1 and 3 min after addition of 1.2 ml TBS was recorded. Enzyme activity was expressed as units, defined as the amount of enzyme giving an increase in absorbance at 490 nm of 0.001 per min.

3.11.7 Antibacterial assay

Nutrient agar plates containing 1% NaCl were used for this assay. The plates were streaked with bacteria with the help of sterile cotton swab. Two fold dilutions of the haemolymph in TBS were prepared for all treatments including control. Whatman filter paper (No.1) discs were soaked in the haemolymph samples and allowed to completely absorb on to the discs. The discs were then placed on the nutrient agar plates and kept for incubation at room temperature. Antibacterial activity was determined by observing the bacterial growth in the plates around the filter paper discs.

3.11.8 Haemagglutinin assay

This assay was performed in V-shaped microwell plates. Two-fold serial dilutions of shrimp haemolymph samples were made in sterile TBS. 100 µl of each dilution was added in to each well of the microtitre plate. An equal volume of 1.5% (v/v) RBC was then added to each well. The plates were incubated at 25±2°C for 1 hr. In negative control, TBS was used instead of haemolymph.

3.11.9 Phagocytosis

Haemolymph immediately after collection was concentrated by centrifugation for 2 min at 2000 rpm at 4°C. 1 or 2 drops of haemolymph was placed on a cover slip and allowed to incubate for 30-35 min. Then, 1 or 2 drops of yeast suspension was added uniformly and incubated for 45 min. It was then fixed with methanol for 2 minutes and stained with Wright's stain for 2 minutes. The slides were then washed with TBS and observed under microscope.

3.11.10 Challenge study

The virulence of the bacterial species to *P. monodon* was checked out in normal shrimps before conducting the challenge study. A bacterial suspension of *V. parahaemolyticus* was prepared in 0.9% saline so as to obtain a concentration of 10^7 cell/ml. Six shrimps from each treatment were taken for the challenge study. 100µl of this suspension was injected per shrimp intramuscularly puncturing dorsally the second abdominal segment.

3.11.11 Survival after challenge

Mortality of the shrimps after experimental challenge with pathogenic *V. parahaemolyticus* was noted in each treatment and percentage survival was calculated as follows:

$$\text{Percentage survival} = \frac{\text{Number of shrimps survived}}{\text{Number of shrimps challenged}} \times 100$$

3.11.12 Colour

Colour of the shrimps in each tank was observed during and at the end of experiment and recorded.

3.11.13 Statistical Analysis

Difference between experimental and control groups were analysed by ANOVA for percentage weight gain, specific growth rate, survival rate, FCR, FCE and phenoloxidase assay at the end of experiment.

Results

4. RESULTS

The effect of different levels of Spirulina in diets on non-specific immune response, growth, survival, colour, food consumption efficiency and food conversion ratio of *Penaeus monodon* was evaluated. The details of the observations made during the study are presented below. The test diets with Spirulina levels 0%, 10%, 20%, 30% and 40% are denoted as T_1, T_2, T_3, T_4 and T_5, respectively.

4.1 Proximate analysis

The proximate composition of the test diets, T_1, T_2, T_3, T_4 and T_5 used in the study, are shown in Table 3. Moisture content of diets ranged between 6.67% to 8.72%, fat content ranged between 7.39% to 8.18% and protein content ranged between 39.15% to 40.55 %. Ash content was between 7.48% to 8.73%. Crude fibre values ranged between 0.43% to 0.51%, whereas carbohydrate ranged between 34.74% to 36.96%.

Table 3: Proximate analysis of feed

Feed	% Moisture	% Fat	% Protein	% Ash	% Crude Fibre	% Carbo hydrates
1.	8.72	8.18	39.15	8.73	0.48	34.74
2..	7.16	7.61	40.10	8.6	0.51	36.02
3.	6.98	8.0	40.55	7.48	0.43	36.56
4.	7.84	7.83	39.80	7.92	0.44	36.17
5.	6.98	7.39	39.55	8.66	0.46	36.96

4.2 Water Quality parameters

The range of temperature, pH, dissolved oxygen and salinity in the experimental tanks during the present study are given in Table 4.

Table 4: Water quality parameters in the experimental tanks during the study period

Parameter		Weeks				
		1	2	3	4	5
Temp	Mean	28	27.833	26.5	27.66	27.166
	Range	27.5–28.4	27–28.4	26-27.5	27-28.2	26.55-27.5
PH	Mean	8	7.83	8	8.16	7.83
	Range	7.5-8.5	7.5-8	7.8-8.5	7.5-8.5	7.5-8
D.O.	Mean	7.0	6.26	6.97	5.8	7.1
	Range	6.9-7.1	6.0-7.1	6.5-7.2	5.8-6.2	7.0-7.2

4.2.1 Temperature

The maximum and minimum temperatures recorded during the study period in the experimental tanks ranged between 26 and 28.4, respectively.

4.2.2 Dissolved oxygen

A minimum of 5.8 ppm and a maximum of 7.2 ppm were recorded in the tanks during the present study.

4.2.3 pH

The pH values during the experimental period varied between 7.5 and 8.5.

4.2.4 Salinity

Salinity of the water was maintained at 20 ppt. during the experimental period.

4.3 Efficiency of various diets.

4.3.1 Percentage weight gain

The data corresponding to the weight gain in the shrimps fed on different diets are given in Table 5. The average live weight gain of shrimps fed on diets with different levels of Spirulina showed maximum growth in treatment T_4 with 30% Spirulina and minimum average growth was shown by the treatment T_1 with 0% Spirulina (Plate 1). The data obtained on the percentage weight gain (Table 5) revealed that the maximum value of 91.10 % was obtained in the treatment T_4 and the minimum of 68.38% was obtained in the treatment T_1. The percentage weight gain of shrimps fed on different diets is presented graphically in Fig 1. According to the analysis of variance the percentage weight gain for the treatments T_2, T_3 and T_4 did not differ significantly. But control and T_5 showed relatively low growth rate (Table 6).

4.3.2 Specific growth rate

The data on SGR are presented in Table 7. The maximum SGR, 1.85% was recorded for the diet with 30% Spirulina level (T_4) and minimum, 1.49% for the diet with 0% Spirulina level (T_1). The SGR of the larvae fed on different diets are graphically represented in Fig 2. Analysis of variance of the data showed that the specific growth rate for the treatments T_2, T_3 and T_4 did not differ significantly. But control and T_5 showed relatively low specific growth rate (Table 8).

Table 5: Growth of *P. monodon* fed on different levels of Spirulina

Treatment	Replication	Av. Initial wt (g)	Av. Final Wt. (g)	Av. Weight gain (g)	% wt. gain	Mean ± SD
T_1	1	1.816	3.036	1.220	67.180	68.375 ± 1.851
	2	1.798	3.020	1.222	67.964	
	3	1.780	2.970	1.190	66.853	
	4	1.825	3.130	1.305	71.506	
T_2	1	1.650	3.162	1.512	91.818	89.466 ± 1.377
	2	1.748	3.294	1.546	88.440	
	3	1.765	3.328	1.563	88.555	
	4	1.699	3.212	1.513	89.052	
T_3	1	1.736	3.312	1.576	90.783	90.962 ± 2.423
	2	1.784	3.339	1.555	87.163	
	3	1.698	3.289	1.591	93.698	
	4	1.745	3.354	1.609	92.206	
T_4	1	1.710	3.277	1.567	91.637	91.104 ± 1.197
	2	1.724	3.281	1.557	90.313	
	3	1.697	3.219	1.522	89.687	
	4	1.718	3.312	1.594	92.782	
T_5	1	1.597	2.761	1.164	72.886	70.583 ± 1.343
	2	1.645	2.793	1.148	69.787	
	3	1.659	2.813	1.154	69.559	
	4	1.699	2.890	1.191	70.100	

Table 6: Analysis of variance of the data on percentage gain in weight of *P. monodon* fed on different diets

Source	d.f.	S.S.	M.S.S	F-ratio
Diets	4	2139.55	534.89	138.93**
Error	15	57.75	3.85	
Total	19	2197.30		

Comparison of treatment means

Critical difference: 2.96

Treatment	T_1	T_5	T_2	T_3	T_4
Means	68.375	70.583	89.466	90.962	91.104

Underscored means are not significantly different

** Significant at 5% level.

Fig. 1 Percentage gain in weight of *P. monodon* juveniles fed on different diets

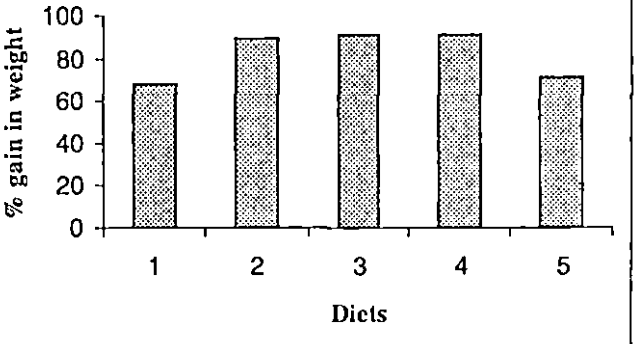


Plate 1. Comparison of growth and colour of *P. monodon* fed on different diets

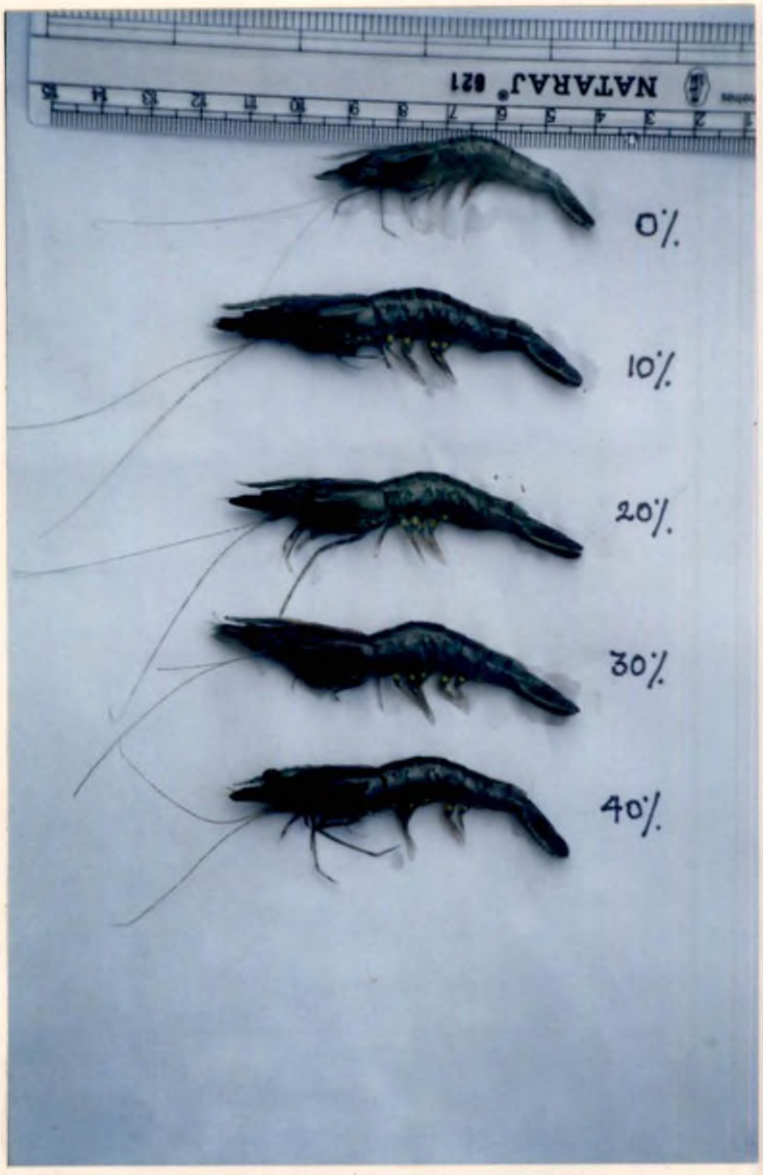


Table 7: Specific growth rate

Treatment	Replication	Av. Initial wt (g)	Av. Final Wt. (g)	Specific growth rate (%)	Mean ± SD
T_1	1	1.816	3.036	1.468	1.488 ± 0.031
	2	1.798	3.020	1.481	
	3	1.780	2.970	1.462	
	4	1.825	3.130	1.541	
T_2	1	1.650	3.162	1.858	1.824 ± 0.019
	2	1.748	3.294	1.810	
	3	1.765	3.328	1.812	
	4	1.699	3.212	1.819	
T_3	1	1.736	3.312	1.845	1.847 ± 0.036
	2	1.784	3.339	1.790	
	3	1.698	3.289	1.888	
	4	1.745	3.354	1.866	
T_4	1	1.710	3.277	1.858	1.85 ± 0.0178
	2	1.724	3.281	1.838	
	3	1.697	3.219	1.829	
	4	1.718	3.312	1.875	
T_5	1	1.597	2.761	1.564	1.525 ± 0.022
	2	1.645	2.793	1.512	
	3	1.659	2.813	1.508	
	4	1.699	2.890	1.517	

Table 8: Analysis of variance of the data on specific growth rate of the *P. monodon* fed on different diets.

Source	d.f.	S.S.	M.S.S	F-ratio
Diets	4	0.5399	0.1349	143.97**
Error	15	0.01406	0.000937	
Total	19	0.5539		

Comparison of treatment means

Critical difference: 0.046

Treatments	T_1	T_5	T_2	T_3	T_4
Means	1.488	1.525	1.824	1.847	1.85

Underscored means are not significantly different

** Significant at 5% level.

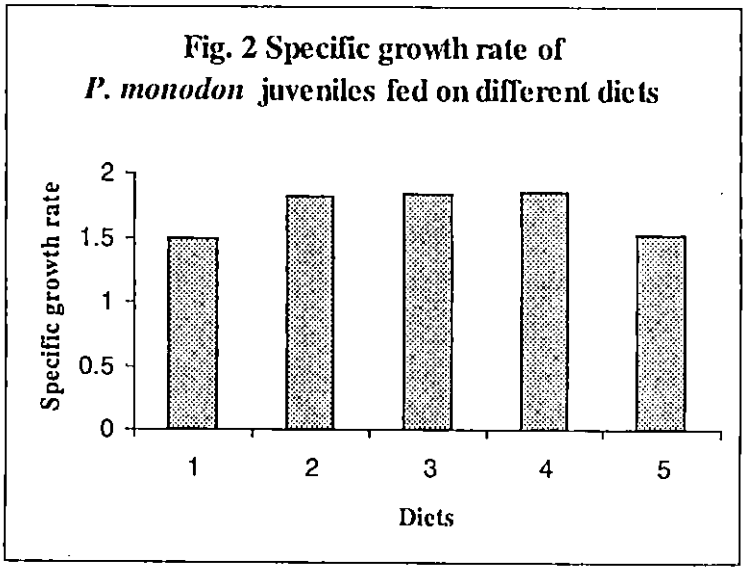


Fig. 2 Specific growth rate of *P. monodon* juveniles fed on different diets

4.3.3 Food Conversion Ratio (FCR)

The data on FCR are represented in Table 9. The maximum FCR, 2.237 was recorded for the diet with 0% Spirulina level (T_1) and minimum, 1.746 for the diet with 20% Spirulina (T_3). The FCR of the shrimps fed on different diets are graphically represented in Fig 3. According to the analysis of variance food conversion ratio for the treatments T_2, T_3 and T_4 did not differ significantly. But control and T_5 showed relatively low food conversion ratio (Table 10).

4.3.4 Food Conversion Efficiency (FCE)

The data on FCE are represented in Table 11. The maximum FCE, 0.57 was recorded for the diet with 20% and 30% Spirulina levels (T_3 and T_4) and minimum, 0.45 for the diet with 0% Spirulina (T_1). The FCE of the shrimps fed on different diets are graphically presented in Fig 4. Analysis of variance of data showed that FCE was significantly higher for treatments T_2, T_3 and T_4 as compared to the treatments T_1 and T_5 (Table 12).

4.3.5 Survival rate at the end of feeding experiment

The percentage survival values of *Penaeus monodon* in various treatments are given in Table 13. The highest average survival was 85% in the treatment T_4 and lowest average survival was 72.5% in the treatment T_1. However, analysis of variance of the data showed that there is no significant difference between the treatments (Table 14). Graphical representation of percentage survival values for the five diets is given in Fig 5.

Table 9: Food Conversion Ratio

Treatment	Replication	Av. Initial biomass	Av. * Final Biomass	Avg. increment	Avg. Feed consumed	FCR	Mean ± SD
T_1	1	1.816	2.408	0.592	1.316	2.222	2.237 ± 0.023
	2	1.798	2.380	0.582	1.326	2.278	
	3	1.780	2.365	0.585	1.298	2.218	
	4	1.825	2.398	0.573	1.280	2.233	
T_2	1	1.650	2.480	0.830	1.426	1.718	1.770 ± 0.042
	2	1.748	2.528	0.780	1.398	1.792	
	3	1.765	2.487	0.752	1.375	1.828	
	4	1.699	2.491	0.792	1.381	1.743	
T_3	1	1.736	2.487	0.751	1.359	1.809	1.746 ± 0.037
	2	1.784	2.613	0.829	1.428	1.722	
	3	1.698	2.485	0.787	1.371	1.742	
	4	1.745	2.586	0.841	1.441	1.713	
T_4	1	1.710	2.497	0.787	1.391	1.767	1.752 ± 0.037
	2	1.724	2.451	0.727	1.311	1.803	
	3	1.697	2.512	0.815	1.386	1.700	
	4	1.718	2.482	0.764	1.328	1.738	
T_5	1	1.597	2.184	0.587	1.239	2.110	2.122 ± 0.017
	2	1.645	2.218	0.573	1.209	2.109	
	3	1.659	2.254	0.595	1.280	2.151	
	4	1.699	2.303	0.609	1.291	2.119	

*** Sampling period - 15 days**

Table 10: Analysis of variance of the data on FCR

Source	d.f.	S.S.	M.S.S	F-ratio
Diets	4	0.8897	0.2224	113.46**
Error	15	0.0295	0.00196	
Total	19	0.9192		

Comparison of treatment means

Critical difference: 0.066

Treatments	T_3	T_4	T_2	T_5	T_1
Means	1.746	1.752	1.77	2.122	2.237

Underscored means are not significantly different

** Significant at 5% level.

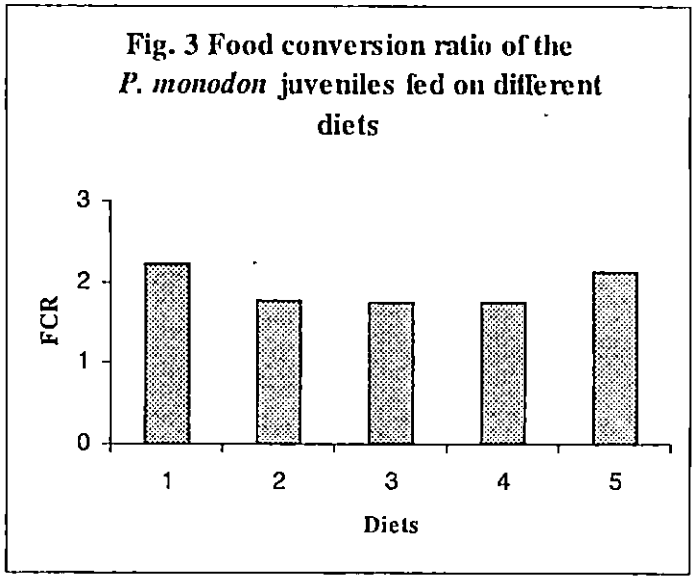


Fig. 3 Food conversion ratio of the *P. monodon* juveniles fed on different diets

Table 11: Food Conversion Efficiency

Treatment	Replication	Food conversion Efficiency*	Mean ± SD
T_1	1 2 3 4	0.449 0.438 0.450 0.447	0.446 ± 0.00474
T_2	1 2 3 4	0.582 0.557 0.546 0.573	0.564 ± 0.0139
T_3	1 2 3 4	0.552 0.580 0.574 0.583	0.572 ± 0.0121
T_4	1 2 3 4	0.565 0.554 0.588 0.575	0.5705 ± 0.0125
T_5	1 2 3 4	0.473 0.473 0.464 0.471	0.470 ± 0.00369

* Sampling period – 15 days

Table 12: Analysis of variance of the data on FCE

Source	d.f.	S.S.	M.S.S	F-ratio
Diets	4	0.0604	0.0151	106.338**
Error	15	0.00213	0.000142	
Total	19	0.06254		

Comparison of treatment means

Critical difference: 0.0696

Treatments	T_1	T_5	T_2	T_4	T_3
Means	0.446	0.470	0.564	0.570	0.572

Underscored means are not significantly different

** Significant at 5% level.

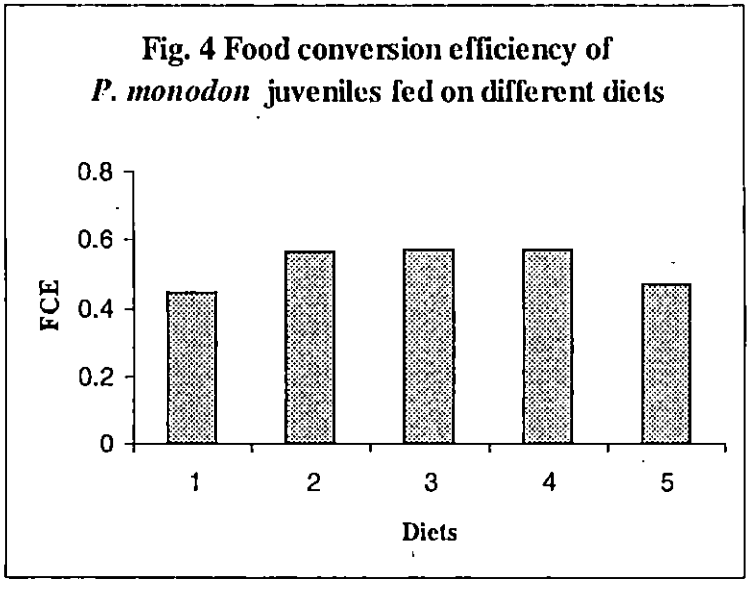


Fig. 4 Food conversion efficiency of *P. monodon* juveniles fed on different diets

Table 13: Survival rate of different treatments at the end of feeding experiment

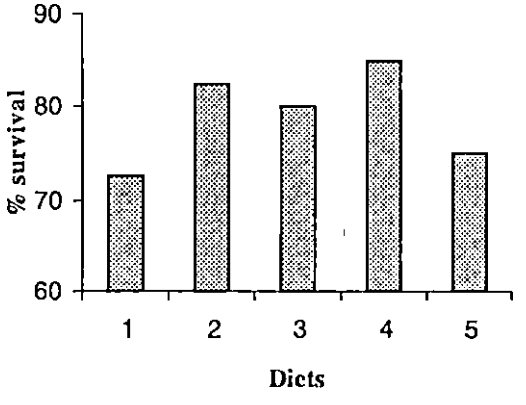
Treatment	Replication	Initial No.	Final No.	%Survival	Mean±SD
T_1	1 2 3 4	10 10 10 10	7 8 7 7	70 80 70 70	72.5 ± 4.33
T_2	1 2 3 4	10 10 10 10	8 8 9 8	80 80 90 80	82.5 ± 4.33
T_3	1 2 3 4	10 10 10 10	8 8 7 9	80 80 70 90	80 ± 7.071
T_4	1 2 3 4	10 10 10 10	10 8 8 8	100 80 80 80	85 ± 8.66
T_5	1 2 3 4	10 10 10 10	8 7 8 7	80 70 80 70	75 ± 5.0

Table 14: Analysis of variance of the data on percentage of survival of *P. monodon* fed on different diets.

Source	d.f.	S.S.	M.S.S	F-ratio
Diets	4	430	107.5	2.15
Error	15	750	50	
Total	19	1180		

Not significant at 5% level

Fig. 5 Percentage survival of *P. monodon* juveniles fed on different diets



4.4 Prophylactic potency of Spirulina

4.4.1 proPhenoloxidase activity

Treatments with Spirulina incorporation at different levels (T_2, T_3, T_4 and T_5) showed significantly higher proPhenoloxidase activity than control (T_1). The data on proPhenoloxidase activity are presented in Table 15. The maximum activity of about 34 enzyme units was recorded for the diet with 40% Spirulina level (T_4) and minimum of 3 units for the diet with 0% Spirulina level (T_1). According to the analysis of variance, proPhenoloxidase activity for the treatments T_2 and T_3 did not differ significantly; T_4 and T_5 also did not differ significantly. But control showed relatively low proPhenoloxidase activity (Table 16). The proPhenoloxidase activity of the shrimps fed on different diets are graphically represented in Fig 6.

4.4.2 Antibacterial assay

All treatments with Spirulina incorporation (T_2, T_3, T_4 and T_5) showed antibacterial activity, observed by the presence of a clear zone around the filter paper discs, indicating the absence of bacterial growth. In control treatment (T_1), no zone could be observed round the filter paper discs (Plate 2A). The clear zone indicating the absence of bacterial growth was marked with the diet T_2 and T_4 (Plate 2B and 2D). Observations of the antibacterial activity are presented in Table 17.

4.4.3 Haemagglutinin assay

Agglutinin activity in shrimp haemolymph was indicated by the absence of button at the bottom of the well of the microtitre plate. All the treatments with Spirulina incorporation (T_2, T_3, T_4 and T_5) showed agglutinin activity at lower dilutions of the haemolymph. Even in the undiluted control haemolymph sample (T_1), no button could be observed. And in 1:2 dilution of the control haemolymph sample too, partial

agglutination was observed. Agglutination was observed up to 1:4 dilution of the haemolymph from shrimp given diet incorporated with 10% Spirulina (T_2). In treatments T_3, T_4, and T_5 similar results were observed. However, at 1:4 dilution only partial agglutination could be observed. Clear buttons were observed in the wells in all the other dilutions of haemolymph. The result of haemagglutinin assay is presented in Table 18.

4.4.4 Phagocytosis

Phagocytic activity was observed in the form of adherence of haemocytes to yeast cell in all treatments with Spirulina incorporation (T_2, T_3, T_4 and T_5). In the treatment T_4, more than one haemocyte could be seen adhering to a yeast cell. In treatments T_2, T_3 and T_5 also, haemocyte adherence was observed much more than was seen in control. In control, adherence of haemocytes to yeast cell was rarely observed. Observations of phagocytic activity are presented in Table 19.

4.4.5 Challenge studies

Vibrio parahaemolyticus found to be pathogenic against *P. monodon* when checked in normal shrimps was used for this study. The mean death times following the challenge of *V. parahaemolyticus* in all treatments with Spirulina was found elevated than the control. Shrimps fed diets with different levels of Spirulina showed delayed mortality compared to control.

4.4.5 Survival after challenge

In control (T_1) there was no survival after 12 hours and mortality reached 100%. In treatments T_2, T_3 and T_4, 100 % mortality was recorded only after a much longer time (20 hours). Surprisingly, even after 24 hours, there was survival in treatment T_5. Survival after challenge with *Vibio parahaemolyticus* is given in Table 20.

Table 15: proPhenoloxidase Activity (Units)

Replications	Enzyme units *				
	T_1	T_2	T_3	T_4	T_5
1	5.5	32	30	34	32
2	6.5	30.	31	32	34
3	5.0	31	29	33	33
4	3.0	32	30	33	34

*Enzyme units – the amount of enzyme giving an increase in absorbance at 490 nm of 0.001 per min

Table 16: Analysis of variance of the data of proPhenoloxidase activity

Source	d.f.	S.S.	M.S.S	F-ratio
Diets	4	2339.5	584.87	551.76**
Error	15	16	1.06	
Total	19	2355.5		

Comparison of treatment means

Critical difference : 1.55

Treatments	T_1	T_3	T_2	T_4	T_5
Means	5	30	31.25	33	33.25

Underscored means are not significantly different

** Significant at 5% level.

Fig. 6 proPhenoloxidase activity (enzyme units)

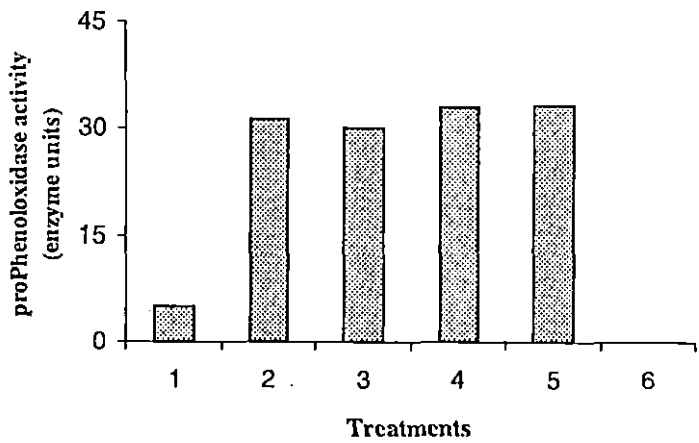


Table 17: Antibacterial Assay

Treatment	Dilution			
	1	2	3	4
T_1	-	-	-	-
T_2	+	+	±	-
T_3	+	±	-	-
T_4	+	+	±	-
T_5	+	±	±	-

+ Zone formation

- No zone formation

± Partial or thin zone

1 Undiluted haemolymph

2 1:2 dilution

3 1:4 dilution

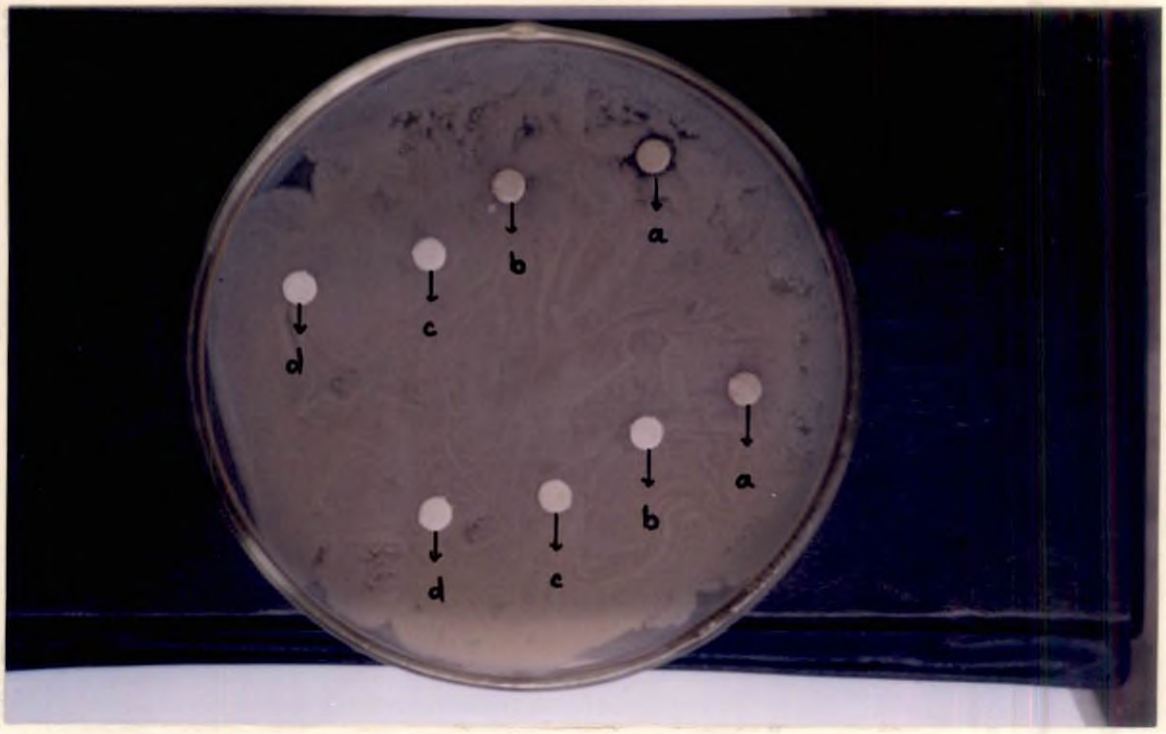
4 1:8 dilution

Plate 2. ANTIBACTERIAL ACTIVITY

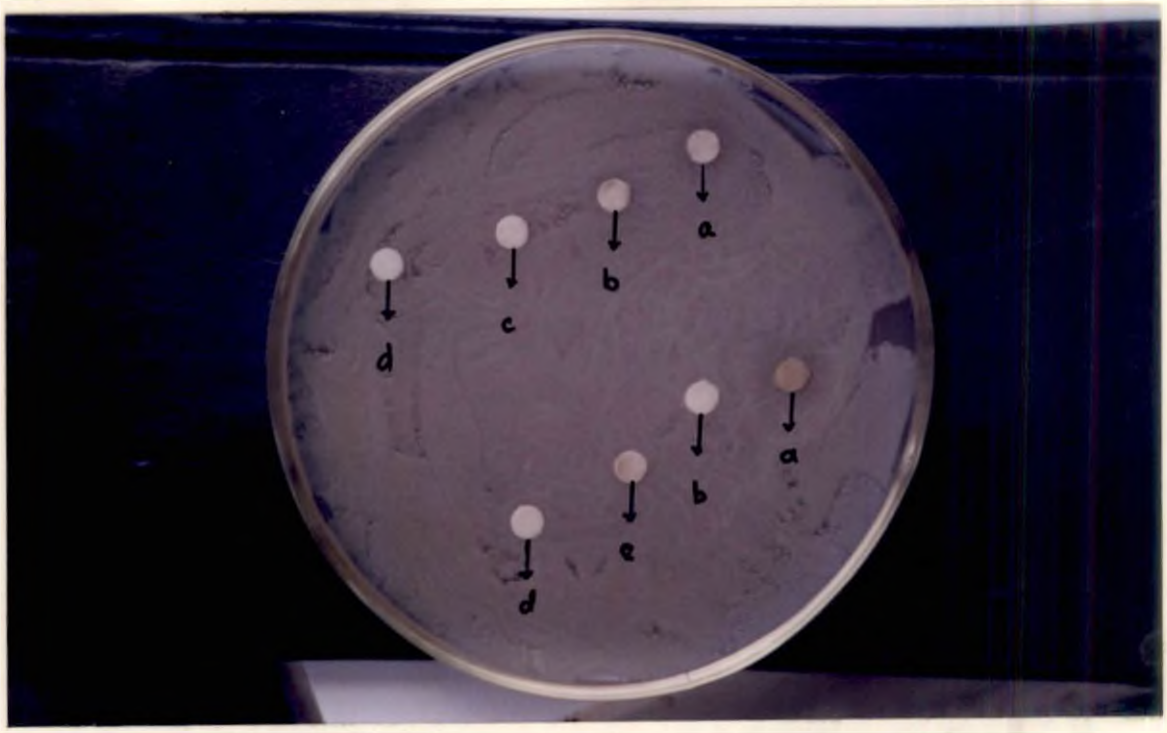
2A. Hemolymph from shrimp fed control diet showing absence of zone around the discs

a. Undiluted haemolymph
b. 1:2 dilution
c. 1:4 dilution
d. 1:8 dilution

2B. Haemolymph from shrimp fed diet with 10% Spirulina, showing clear zone (absence of bacterial growth) around the discs

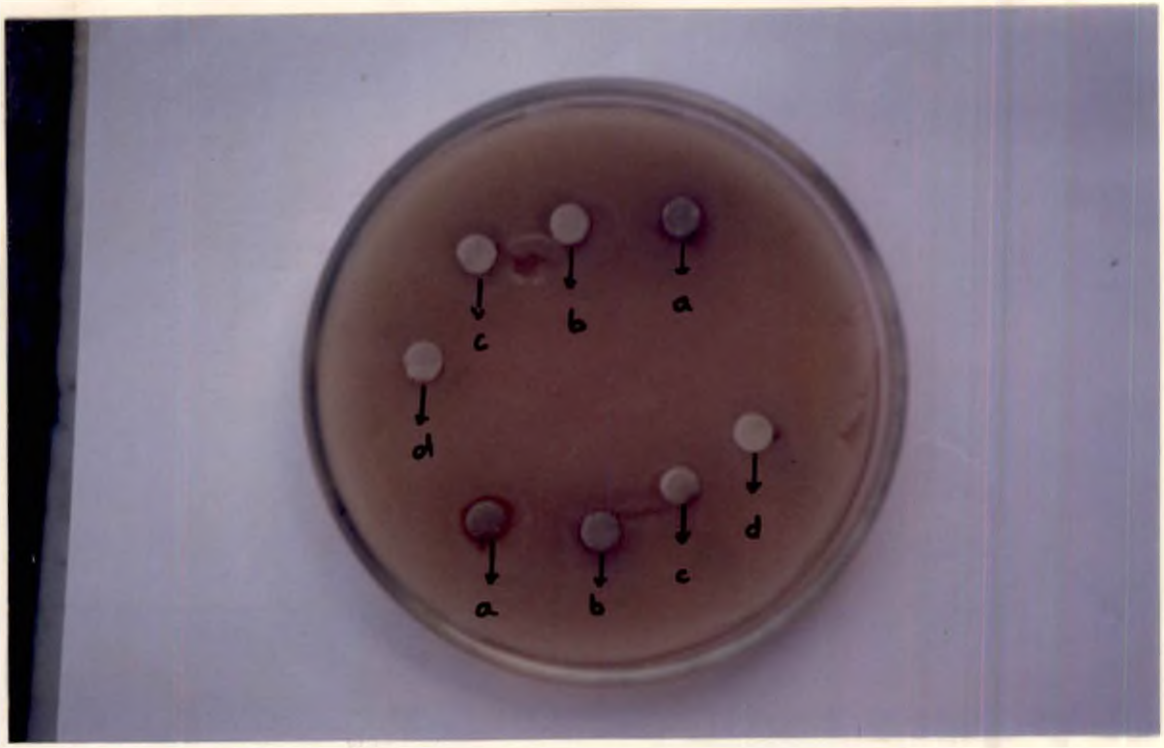


2C. Haemolymph from shrimp fed diet with 20% Spirulina, showing zone (absence of bacterial growth) around the discs

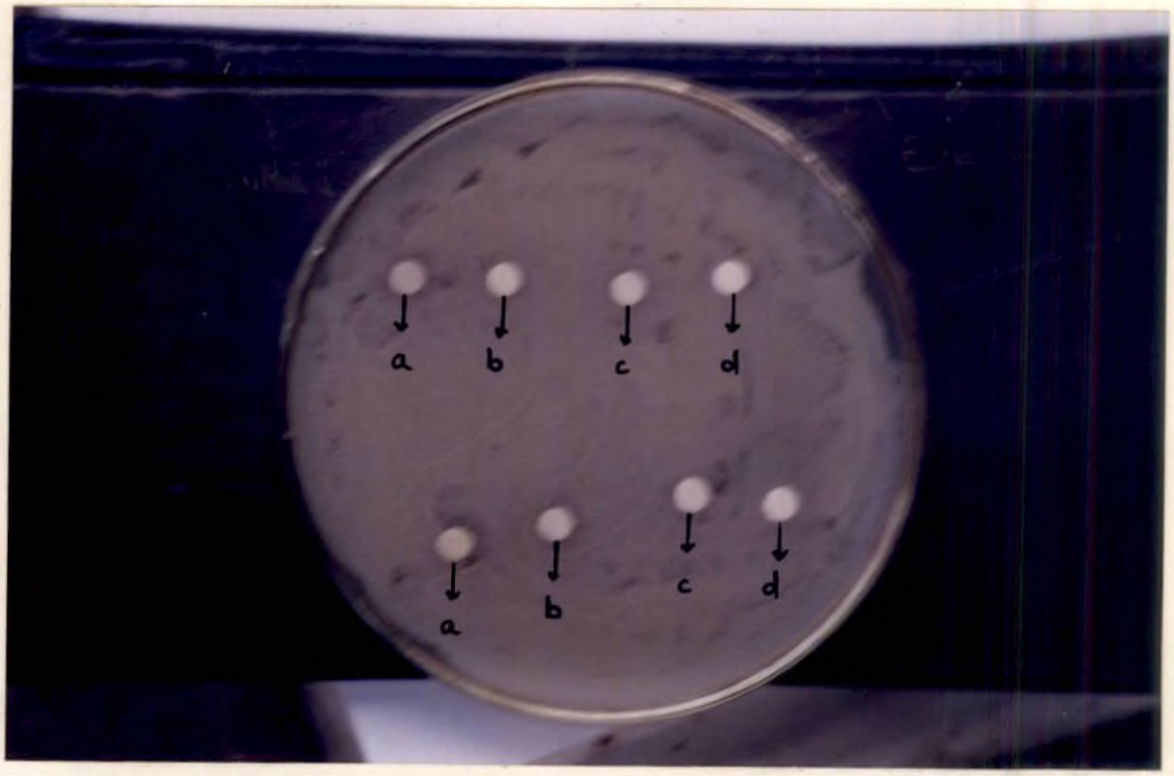


a. Undiluted haemolymph
b. 1:2 dilution
c. 1:4 dilution
d. 1:8 dilution

2D. Haemolymph from shrimp fed diet with 30% Spirulina, showing clear zone (absence of bacterial growth) around the discs



2E. Haemolymph from shrimp fed diet with 40% Spirulina, showing zone (absence of bacterial growth) around the discs



a. Undiluted haemolymph
b. 1:2 dilution
c. 1:4 dilution
d. 1:8 dilution

Table 18: Haemagluttinin Assay

Treatment	Dilution				
	1	2	3	4	5
T_1	+	±	-	-	-
T_2	+	+	+	-	-
T_3	+	+	±	-	-
T_4	+	+	±	-	-
T_5	+	+	±	-	-

+ Agglutination

- No Agglutination

± Partial Agglutination

1 Undiluted haemolymph

2 1:2 dilution

3 1:4 dilution

4 1:8 dilution

5 1:16 dilution

Table 19: Phagocytosis

Treatment	Observations
T_1	Very few haemocytes were adhered to yeast cell.
T_2	More than one haemocyte were adhered to one yeast cell.
T_3	Haemocytes were adhered to yeast cell.
T_4	More than one haemocyte were adhered to one yeast cell.
T_5	Many haemocytes were adhered to the yeast cells.

Table 20: Effect of *Vibrio parahaemolyticus* challenge on *P. monodon*, expressed as cumulative mortality (Percentage survival of total given in parentheses) (n = 6)

Treatments	Time in hours after challenge					
	4hours	8 hours	12 hours	16 hours	20 hours	24 hours
T_1	1(83.3)	4(33.3)	6(0)	-	-	-
T_2	0(100)	1(83.3)	3(50)	4(33.3)	6(0)	-
T_3	0(100)	1(83.3)	3(50)	4(33.3)	6(0)	-
T_4	0(100)	1(83.3)	2(66.6)	4(33.3)	6(0)	-
T_5	0(100)	1(83.3)	2(6.66)	4(33.3)	4(33.3)	5(16.6)

4.4.6 Colour

Colour of the shrimps treated with Spirulina and control was observed. Shrimps treated with Spirulina (T_2, T_3, T_4 and T_5) showed darker colouration than control (T_1) (Plate 1). Colour observations of shrimps are shown in Table 21.

Table 21: Colour variations of *P. monodon* in various treatments

Treatment	Observations
T_1	+
T_2	+++
T_3	++
T_4	+++
T_5	+++

\+ Normal colour, lighter than Spirulina treated shrimps.

++ Darker colouration than control.

+++ Very dark colouration .

Discussion

V. DISCUSSION

5.1 Proximate analysis of formulated pelleted feeds

The protein requirement of *Penaeus monodon* has been identified to be 35% by Bages and Sloane (1981). Bautista (1986) reported that 40% to 50% protein gave best growth and survival of *P. monodon* in the presence of 20% carbohydrate and 5% to 10% lipid. Proximate analysis of the diets used in the present study revealed that they contained protein in the range of 39.15%-40.55%, which is almost the same as the optimum value suggested by previous workers.

The diets used in the present study contained carbohydrates in the range of 34.74%- 36.96%. Bages and Sloane (1981) obtained high growth rate in *P. monodon* with diet containing 20% carbohydrate. But Catacutan (1991) did not find any difference in growth of juvenile *P.monodon* fed isonitrogenous diets containing 5%-35% carbohydrate.

Andrews *et al.* (1972) reported that lipid level below 10% was adequate in shrimp diets. Bautista (1986) observed that excessive dietary lipid has an adverse effect on the growth and survival of shrimp. The lipid content of the diets used in the present study is in the range of 7.39%- 8.18%. The ash content was worked out to be varying between 7.48%- 8.73%.

5.2 Water quality parameters

5.2.1 Temperature

A temperature range of 28 ± 2°C has been found to be optimum for *Penaeus monodon* growth (Foster and Beard, 1974). Several workers have reported wide temperature tolerance for *P. monodon* (Liao, 1977; Sasai, 1981; Chen *et al.*, 1985). Chakraborti *et al.* (1986) observed a temperature tolerance of 24°C- 30.5°C for this species. The

weekly range of temperature observed during the present study was 26.5°C to 28.5°C. The values recorded are within the optimum range suggested for the growth of *P. monodon*. The temperature fluctuation was gradual and could be maintained uniformly throughout the experimental period since the tanks were housed indoor.

5.2.2 Dissolved oxygen

Chakraborti *et al.* (1985) reported that the dissolved oxygen below 2.5 ppm affected the growth and survival of *P. monodon*. Chakraborti *et al.* (1986) have suggested the optimum range to be 6.8 – 7.6 ppm for *P. monodon* though they can tolerate dissolved oxygen as low as 4.8 ppm. Studies made by Liao and Huang (1975) and Chen *et al.* (1985) revealed that the oxygen consumption of post larvae of *Penaeus monodon* decreased whenever dissolved oxygen fell below 3.8 and 4.0 ppm. The weekly dissolved oxygen values in the experimental tanks ranged from 5.8 to 7.1 ppm. Since aeration was not provided in the tanks. These values were found to be optimum for the growth of *P. monodon* juveniles.

5.2.3 pH

Chakraborti *et al.* (1986) obtained maximum growth rate at pH 8.4 – 8.7. Noor Hamid *et al.* (1992) recommended a near neutral pH of 7.6 – 8.0 for faster growth. In the present experiment, the weekly range of pH value in the tanks was from 7.5 to 8.5. These values conform to those obtained in the previous studies and are within the optimum range.

5.2.4 Salinity

Sundararajan *et al.* (1979) and Navas (1988) did not find any significant difference in growth of *P. monodon* post larvae reared at 4.5 and 15 ppt with those reared at 20 ppt. Accordingly, the salinity

maintained in the experimental tanks for rearing *P. monodon* juveniles kept at 20 ppt is the optimal level for this species.

5:3 Growth studies

5.3.1 Feeding experiment

In the present study, efficiency of Spirulina in replacing clam meat at different levels in the diet for *Penaeus monodon* juveniles was tested based on percentage weight gain, specific growth rate, survival, food conversion ratio and food consumption efficiency.

5. 3. 1.1 Percentage weight gain

Growth of *Penaeus monodon* in different treatments indicated that shrimp juveniles fed on the diet T_4 containing 30% Spirulina gave the maximum average live weight gain, followed by T_3 containing 20% Spirulina and T_2 containing 10% Spirulina. Statistically there was no significant difference between treatments T_2, T_3 and T_4. The lowest growth performance was recorded in T_1 containing 0% Spirulina. It was observed that with the increase of Spirulina content to 40% (T_5), the growth of *P. monodon* juveniles decreased significantly (Table 4 and Fig.1). The results indicate that Spirulina can replace clam meat up to 30% level in the diet of *P. monodon* juveniles. Though a number of reports on replacement of fishmeal with non-conventional plant feed stuffs are available (Santiago, *et. al.*, 1988; Ng and Wee, 1989; De Silva and Gunasekera, 1989), it appears that for most of the non-conventional plant feed stuffs, the maximum recommended level of inclusion appears to be between 20 and 30% of diets. Reports of fishmeal replacements from practical fish diets with single cell protein and leaf protein concentrates were also made by Appler and Jauncey (1983); Davies and Wareham (1988). The maximum recommended level of inclusion in the

above reports were also found to be 20 to 35%. Chow and Woo (1990) recommended a 20% replacement of fishmeal with Spirulina in the diet for *Oreochromis mossambicus*.

Studies on the effect of different immunostimulants on growth showed that use of immunostimulants have a positive effect on growth of fish/shrimps, as was observed in the present study. For instance, when levamisole was used as an immunostimulant in marine teleost gilthead seabream (*Sparus auratus* L.), the specimens from treated groups were larger and heavier than those of the control group (Mulero *et al.*, 1998). Similarly in shrimps, Sung *et al.* (1994) observed enhanced growth in *Penaeus monodon* immersed in glucan. Boonyaratpalin *et al.* (1995) also reported that black tiger shrimp fed with peptidoglycan supplemented feed showed better growth than those fed a normal diet. Recently, Azad *et al.* (2002) too found that the final average weight of the harvested shrimp immunostimulated with whole cell preparations of *Vibrio* was significantly higher than that of control shrimps.

5.3.1.2 Specific growth rate

Specific growth rate can be considered as an index of growth in the evaluation of diets. The results of the present study indicate that the highest specific growth rate was obtained with the diet T_4 with 30% Spirulina, followed by T_3 with 20% Spirulina and T_2 with 10% Spirulina. Statistically there was no significant difference among these three diets. The comparatively better specific growth rate recorded with these three diets indicates their better utilization and efficient conversion compared to the control diet (T_1) where only clam meat was used (0% Spirulina). Immunostimulants are known to increase transport and utilization of certain specific micronutrients such as vitamins, pigments and other important compounds (Dugger and Jory, 1999). This might be one of the reasons for growth enhancement by use of immunostimulants.

Spirulina used as an immunostimulant in the present study is a rich source of proteins, vitamins, irons and carotenoid pigments (Venkataraman, 1983), which may together have contributed to the high specific growth rate, observed in the present study with diets incorporating 10-30% levels of Spirulina.

Manju (1994) reported that the Spirulina protein level of 35% was the optimum level for supporting maximum specific growth in *Etroplus suratensis*. Matty and Smith (1978) also evaluated Spirulina as the sole source of protein for rainbow trout and reported that 35% protein diet produced the highest specific growth rate.

However, in the present study, the effect of Spirulina incorporation at 35% level was not tested. By using Spirulina as the major protein source at 40% level of incorporation, by complete replacement of clam meat, comparatively less growth rate was obtained, suggesting that some amount of animal protein in the diet may be essential for optimum growth in shrimps. Similarly, when *Macrobrachium rosenbergii* was fed with *Spirulina fusiformis*, it was observed that Spirulina cannot serve as the sole protein source in the diet, but can be effectively used as a supplementary protein (James *et al.*, 1992).

5.3.1.3 Food conversion ratio (FCR) and food conversion efficiency (FCE)

Food conversion ration (FCR) is the ratio of food consumed by the animal to the live weight it has gained. It indicates the efficiency with which an animal can convert food for the growth process. Whereas food conversion efficiency is the ratio of live weight gained by the animal to the feed it has consumed. Thus, low food conversion ratio indicates high food conversion efficiency, or in other words, better food utilization. In the present study, there was no significant difference in

FCR and FCE values of treatments T_2, T_3 and T_4 with 10%, 20% and 30% Spirulina incorporation, respectively. The FCR was lower and FCE was higher in these treatments than the control diet T_1. The highest FCR of 2.24 and lowest FCE of 0.45 was obtained for the control diet T_1 with 0% Spirulina. This result correlates well with the results of specific growth rates obtained in the different treatments, in the present study. Boonyaratpalin *et al.* (1995) too observed that black tiger shrimp fed with peptidoglycan supplemented feed showed better feed conversion ratio than those fed a normal diet. However, when Spirulina level was increased to 40% FCR increased and FCE decreased suggesting poor utilization and assimilation of feed. Manju (1994) also reported that FCR increased when percentage of Spirulina protein in the diet increased above 35% when fed to *Etroplus suratensis*.

5.3.1.4 Survival during experiment

In the present study, maximum survival was observed in treatment T4, where *P. monodon* juveniles were fed with pelleted feed containing 30% Spirulina. However, survival rate of the juveniles was not significantly different among dietary treatments. Carps treated with levamisole showed no significant difference in survival when compared with control (Siwicki and Korwin-Kossakowshi, 1988). Manju (1994) also reported that when *Etroplus suratensis* was fed with Spirulina incorporated diet, survival rate of the fingerlings was not significantly different among dietary treatments. Song *et al.* (1997) also observed that survival rate of *P. monodon* treated with glucan was not significantly different from the control animals. Similarly, when shrimps were treated with *Vibrio* whole cell preparations, there was no significant difference between the survival rate of control and immunostimulated shrimps (Azad *et al.*, 2002).

5.4 Prophylactic potency of Spirulina

The crustacean host defense is largely based on the activities of the haemocytes and haemolymph factors. The haemocytes are capable of phagocytosis, encapsulation, nodule formation and mediation of cytotoxicity. The proPhenoloxidase system is an important mediator of the crustacean immunity (Soderhall and Cerenius, 1992).

The haemolymph factors include naturally occurring or inducible bioactive molecules, which agglutinate, precipitate or inactivate non self-particles and those that have bacteriolytic or bacteriostatic properties (Karunasagar and Karunasagar, 1999). In the present study, an attempt has been made to study some of these important components, which are indicators of the non-specific defense system in shrimps, after feeding *Penaes monodon* juveniles with diets incorporated with different levels of Spirulina.

5.4.1 Phenoloxidase activity

Shrimps fed with Spirulina incorporated diet (T_2, T_3, T_4 and T_5) showed enhanced phenoloxidase activity as compared to control. Statistical analysis showed significant difference between Spirulina incorporated treatments and control. Highest enzyme activity was observed with 40% Spirulina (T_5) and lowest with 0% Spirulina (T_1). *In vitro* beta-glucan treatment also enhanced the phenoloxidase activity in *P. monodon* (Sung, *et. al.*, 1994). Enhanced activity of proPhenoloxidase system *in vitro* was also reported by Sritunyalucksana *et al.* (1999) when peptidoglycan and lipopolysaccharide (LPS) were added to the haemocyte lysate fraction (HLF) of *P. monodon*. Sung and Sun (1999) also observed enhanced proPO activity in *P. monodon* following the treatment with beta-glucan, zymosan and *Vibrio* antigen. Baruah and Paniprasad (2001) also observed that proPhenoloxidase

system of *Macrobrachium rosenbergii* treated with levamisole was significantly higher than the control.

The proPO system participates in defense in a number of ways. This may lead to production of microbicidal compounds such as quinolones and melanin. They may also interact with other proteins and mediate haemocyte adherence and stimulate encapsulation of large particles by granular cells (Soderhall and Cerenius, 1992).

5.4.2. Antibacterial assay

Haemolymph samples from shrimp treated with Spirulina showed antibacterial activity against *Vibrio parahaemolyticus* in the *in vitro* study conducted. The zones around the filter paper discs indicating the inhibition of bacterial growth, was most clear with the diets T_4 and T_2 containing 30% and 10% Spirulina, respectively. The inhibition of bacterial growth around the haemolymph samples treated with Spirulina may be due to the stimulation of bactericidins in the haemolymph or due to generation of melanin and its intermediaries generated through proPO system, which have been shown to have antimicrobial activity. Bactericidins have been described in crabs and lobsters also (Karunasagar and Karunasagar, 1999). When salmonids were treated with lysozymes they also showed enhanced antibacterial activity against furunculosis (Siwicki *et al.*, 1998).

5.4.3 Haemagglutinin assay

Agglutinins, which cause aggregation or agglutination of foreign particles, have been reported from a number of crustacean species (Karunasagar and Karunasagar, 1999). Though agglutinins occur naturally, enhanced titres after exposure to test materials has been reported for *P. monodon* (Adams, 1991). However, the effect was small, short-lived and non-specific. Agglutinins would help sequestration of

invasive organisms from the haemolymph and therefore would contribute to disease resistance. Lectins, which are generally recognized by their activity to cause haemagglutination, are also found in many crustacean species. The function of these haemaogglutinins in defense is not understood. It is suspected that lectins serve as recognition molecules (Karunasagar and Karunasagar, 1999).

In the present study, shrimps treated with Spirulina showed agglutinin activity upto 1:4 dilution of haemolymph. However, in control haemolymph (T_1) also agglutinin activity was observed in undiluted and 1:2 dilution of haemolymph. This suggests that there is agglutinin activity in normal shrimps also. Hence, further study is needed to understand the agglutinin activation property of Spirulina in *P. monodon*.

5.4.4 Phagocytosis

Phagocytosis is the most important cellular defense reaction and together with humoral components, constitutes the first line of defense once a pathogen has overcome the physiological barrier of the cuticle. Foreign particles such as bacterial cells can be removed by phagocytosis or by haemocyte encapsulation, which is initiated by the proPhenoloxidase system (Soderhall and Cerenius, 1992). In the present study, when shrimps were fed with Spirulina through feed, haemocytes showed increased adherence to yeast cells in comparison with the haemocytes from control diet fed shrimp. These results are similar to the results obtained by Itami *et al.* (1994), where haemocytes from shrimp treated with β-glucan showed higher phagocytic activity than haemocytes from control diet fed shrimp. Itami *et al.* (1998) also showed that when Kuruma shrimp, *Penaeus japonicus*, were orally administered with peptidoglycan derived from *Bifidobacterium themophilium* the haemocytes showed increased phagocytosis as

compared to haemocytes from control animals. Yoshida *et al.* (1993) also showed similar results where rainbow trout fed with EF203 had higher phagocytic activity than control.

In the present study, increased adherence of haemocytes from Spirulina treated shrimp to yeast cells shows that Spirulina can enhance the phagocytic activity in the shrimp haemocytes.

5.4.5 Challenge study

In the present study, shrimps treated with Spirulina incorporated diet (T_2, T_3, T_4 and T_5) showed delayed mortalities than the control diet fed shrimps when challenged with *Vibrio parahaemolyticus* bacteria. Delayed mortality in shrimps treated with Spirulina suggests that Spirulina can act as an immunostimulant and can provide protection against bacterial infection. A few workers have reported enhanced survival of shrimp treated with immunostimulants when challenged with bacterial or viral pathogens. Itami *et al.* (1989) noted that cultured kuruma prawns treated with *Vibrio* bacterin showed reduced mortalities compared to untreated control when challenged by *Vibrio* injection 30 days later. Delayed mortality was also observed in *Macrobrachium rosenbergii* treated with levamisole on challenge with a virulent strain of *Pseudomonas fluorescens* (Baruah and Paniprasad, 2001).

5.4.6 Colour

In the present study, *P. monodon* juveniles treated with Spirulina (T_2, T_3, T_4 and T_5) showed darker colouration than control diet fed juveniles. This indicates the high quality carotene in the Spirulina. James *et al.* (1992) also reported good pigmentation of *M. rosenbergii* treated with *Spirulina fusiformis*.

From the observed results, it can be concluded that Spirulina can be used as an immunostimulant in tiger shrimp, *P. monodon*. The only demerit of using the processed form of Spirulina is the cost of feed. But if we consider the huge loss encountered in shrimp culture due to disease outbreaks, the high cost of feed may not be a critical factor, especially if we take into account the enhanced growth rate and improved colour of Spirulina fed shrimps. The results of the present study indicates that 10% level of incorporation of Spirulina in feed can be recommended, as there is no significant difference among 10%, 20% and 30% level of incorporation.

Although results obtained from this study suggests the suitability of Spirulina as an immunostimulant in *P. monodon*, further studies are needed using unprocessed Spirulina, to determine the optimum level of incorporation of Spirulina into feed to make its use economical. Since the effect of long-term administration of immunostimulants is still unknown, further studies are also warranted for determining the effective duration of administration.

Summary

VI. SUMMARY

1. The objective of the present study was to assess the immunostimulating potential of Spirulina given through feed and to study the effect of Spirulina incorporation in feed on growth and colour of *Penaeus monodon*.

2. Juveniles of *P. monodon* having an average weight of 1.7g were used as experimental animals. The experiment was conducted in completely randomized design with five treatments and four replications, each for a period of 35 days.

3. For the study, five diets with 40% protein were prepared by replacing clam meat with Spirulina. They were diet T_1 with 0% Spirulina, diet T_2 with 10% Spirulina, diet T_3 with 20% Spirulina, diet T_4 with 30% Spirulina and diet T_5 with 40% Spirulina. The ingredients other than Spirulina and clam meat were groundnut oil cake, rice bran, cod liver oil and vitamin-mineral mix.

4. Water quality parameters in the experimental tanks were monitored at weekly intervals and the variations observed in the water quality parameters were found to be well within the tolerance limit of *P. monodon* juveniles.

5. At the end of 35 days of rearing, the highest growth rate was observed in the treatment T_4 with 30% Spirulina and lowest growth was observed in the treatment T_1 with 0% Spirulina. However, no

statistically significant difference was observed among treatments T_2, T_3 and T_4. But T_1 and T_5 showed relatively low growth rate.

6. The lowest food conversion ratio (FCR) of 1.75 was recorded in shrimps juveniles fed on diet T_3 with 20% Spirulina and T_4 with 30% Spirulina, while highest FCR was recorded for diet T_1 (2.24) with 0% Spirulina. The lowest food conversion efficiency (FCE) of 0.45 was observed for the diet with 0% Spirulina, while highest FCE (0.57) was recorded for treatments T_3 (20% Spirulina) and T_4 (30% Spirulina). Statistically no significant difference was observed in FCRs and FCEs of treatments T_2, T_3 and T_4. But treatments T_1 and T_5 showed relatively high FCR, relatively low FCE.

7. There was no statistically significant difference observed in the survival of shrimps fed on the different dietary treatments.

8. Indices used for assessing immunostimulating potential of Spirulina were prophenoloxidase activity, antibacterial assay, haemagglutinin assay, phagocytosis and challenge studies.

9. Spirulina incorporated treatments (T_2, T_3, T_4 and T_5) showed significantly higher phenoloxidase activity than control.

10. All treatments with Spirulina incorporation (T_2, T_3, T_4 and T_5) showed antibacterial activity, while in control it was not observed.

11. Agglutinin activity was observed in the treatments incorporated with Spirulina up to 1:4 dilution of haemolymph, while in control also

agglutinin activity was observed in neat haemolymph (without dilution) and 1:2 dilution (partial agglutination).

12. Increased phagocytosis in the form of adherence of haemocytes to yeast cells was observed in treatments incorporated with Spirulina than control.

13. The mean death time following the challenge with *Vibrio parahaemolyticus* in all the treatments with Spirulina was found elevated than the control. 100% mortality was observed in control after 12 hours, while in treatments with Spirulina 100% mortality was observed after much longer time (20 hours). There was survival in treatment T_5 even after 24 hours.

14. Spirulina fed shrimps showed darker colouration than the control diet fed shrimps.

15. From the study it is clear that Spirulina can be used as an immunostimulant in *P. monodon* with additional benefits of growth promotion and colour enhancing properties. But it needs further study to determine the exact level of incorporation of Spirulina.

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BIOMODULATION OF NON-SPECIFIC IMMUNE RESPONSE IN THE TIGER SHRIMP *PENAEUS MONODON* FABRICIUS WITH SPIRULINA INCORPORATED DIET

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ABSTRACT OF THE THESIS

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ABSTRACT

Black tiger shrimp; *Penaeus monodon* is an important species cultured all over the world. However, disease outbreaks have caused serious economic losses in several countries. For successful farming of shrimps disease prevention is a prime necessity. The present study was designed to assess the immunostimulating potential of Spirulina and to see its effect on growth as well as colour of *P. monodon* juveniles.

In this study, juveniles of *P. monodon* were used for evaluating the effect of different levels of Spirulina on growth, food conversion ratio, food conversion efficiency, survival as well as prophylactic potency of Spirulina evaluated by proPhenoloxidase activity, antibacterial assay, haemagglutinin assay, phagocytosis and challenge study. Effect of Spirulina on colour of shrimps was also studied. Five experimental diets designed as T_1 to T_5 were prepared by using clam meat, Spirulina, rice bran, cod liver oil and vitamin-mineral mix. The total protein content of all diets were kept near to 40% and Spirulina was incorporated at 0%, 10%, 20%, 30% and 40% in diets by replacing clam meat. Completely randomized design with five treatments each having four replications was used for analyzing the results. In each tank ten numbers of *Penaeus monodon* juveniles were kept. Feeding was done for a period of 35 days.

The test animals fed with diet containing Spirulina up to 30% incorporation showed better growth, food conversion ratio and food conversion efficiency. But survival during experiment was not affected by the addition of Spirulina. Spirulina incorporated treatments showed higher phenoloxidase activity than control. Antibacterial activity was also observed in the treatments incorporated with Spirulina, while in control no antibacterial activity was observed. Result of agglutinin activity was not

clear as control also showed agglutination in undiluted haemolymph sample and partial agglutination in 1:2 dilution of haemolymph. Spirulina incorporated treatments showed agglutination up to 1:4 dilution of haemolymph. But in 1:4 dilution only partial agglutination was observed. Increased adherence of haemocyte to yeast cells was observed indicating higher phagocytic activity in the treatment incorporated with Spirulina, while adherence of haemocyte was rarely observed in control. On challenge with *Vibrio parahaemolyticus*, shrimps treated with Spirulina incorporated diet showed delayed mortality as compared to control.

Spirulina treated shrimps showed darker colouration than control.