CHAPTER – XII

EFFECT OF IMMUNOSTIMULANTS ON LARVAL GROWTH, SURVIVAL AND DISEASE RESISTANCE

Shrimp production from aquaculture has plummeted worldwide due to disease outbreaks and has caused serious economic losses. Shrimp culture from its hatchery level has now become unpredictable because of mass mortality at all stages, suboptimal growth, poor disease resistance of larvae etc. Although, bacteria, parasites, fungi and viruses cause disease problems in commercial hatchery, experience has shown that bacteria present in hatcheries are the main cause of the problem. As the reported disease incidence due to specific pathogens are low (Munro *et al.*, 1995), the opportunistic bacteria that benefit from enriched cultivation conditions proliferate in the water, live food or gut of larvae or juveniles/adults are considered as the reason. Adding to this, in hatcheries, infected broodstock is a good source of pathogens, transmitting the same through eggs to nauplii (vertical transmission).

Ability to control occurrence of pathogenic bacteria in the intensive larval rearing of penaeid shrimp has been a major factor affecting production success and larval quality. Various hatchery management practices are aimed at controlling the opportunistic pathogens, either by water disinfection or/and hygienic practices and by using antibiotics. As these methods tend to destabilize the bacterial populations, in the long run provide only a temporary respite from the problem. For example, chlorine is widely used in hatcheries and ponds, but its use stimulates the development of multiple antibiotic resistance genes in bacteria (Murray *et al.*, 1984).

The long-term use of antibiotics may result in the development of antibiotic resistant pathogens (Husevag *et al.*, 1991; Anderson and Sandaa, 1994). According to these authors, not only resistant bacteria proliferate after an antibiotic has killed off the other bacteria, but also they can transfer their resistance genes to other bacteria that have never been exposed to the antibiotic. The subtherapeutic (prophylactic) use of antibiotics (related to human medicine) or the use of any antimicrobial agent known to select for cross-resistance to antimicrobials used in human medicine could pose a particularly significant hazard to human health (Salyers, 1995; Witte *et al.*, 1999).

Several alternative strategies to the use of antimicrobials in disease control have been proposed and have already been applied very successfully in aquaculture. A strategy for disease control, in addition to improving environmental conditions, must also include measures to increase the larval resistance to infection. Enhancing the nonspecific defense mechanisms of the host by immunostimulants, alone or in combination with vaccines, has been sighted a very promising approach (Raa, 1996; Sakai, 1999; Skjermo and Vadstein, 1999; Skjermo *et al.*, 2001). An immunostimulant may be defined as an agent that stimulates the nonspecific immune mechanisms when given alone, or specific mechanisms when given with an antigen (Vadstein, 1997).

In hatcheries these products can be administered to larval stages by oral, immersion or bioencapsulation and delivery via live feed organisms. These products are considered to stimulate non-specific immune responses and augment disease resistance, improve survival and growth of treated animals, thus enhancing production (Robertson *et al.*, 1990; Rorstad *et al.*, 1993; Verlhac *et al.*, 1998: Raa, 2000). Many different types or group of substances are known to act as immunostimulants (Yano *et al.*, 1991; Anderson, 1992; Anderson and Siwicki, 1994; Raa, 1996; Mulero *et al.*, 1998).

Positive results with immunostimulants have been reported with adult as well as larval finfish (review by Vadstein, 1997 and Sakai, 1999; Skjermo *et al.*, 2001; Sahoo and Mukhergee, 2001). Controlled laboratory studies have demonstrated that immunostimulants such as yeast glucan, peptidoglucan, schizophyllum, lipopolysaccharide, chitosan and levamisole have the potential to reduce the impact of disease in shrimp (Itami *et al.*, 1989; Sung *et al.*, 1994; Song and Hsieh, 1994; Itami *et al.*, 1994; Sritunyalucksana *et al.*, 1999). Crustaceans treated with immunostimulants such as glucan (Kenkyu, 1994; Chang *et al.*, 1999), levamisole (Baruah and Prasad, 2001) usually show enhanced defence cell activities. In tiger shrimp *Penaeus monodon*, increased bacterial clearance was noted after injection with glucan (Sung *et al.*, 1996).

It is worth mentioning that only limited number of studies have been carried out with the use of immunostimulants in early life stage of shrimp. Moreover, most of the studies were restricted to postlarval stages and juveniles. Establishment of an immunostimulation strategy should include evaluation, administration procedures, dose-response relationships and evaluation of the duration of stimulation (Vadstein, 1997). This experiment was carried out for the development of application strategy for three immunostimulants (two natural and one synthetic) by studying the above factors. In addition to the usual evaluation through challenge tests, the effect of the stimulants on growth and survival were also studied. Failure to obtain response after *in vivo* immunostimulation is often blamed on suboptimal exposure times or doses (Skjermo *et al.*, 2001). Moreover, over-dosing and long-term administration of such products may reduce their efficacy and cause immunosuppression and growth retardation (Sakai, 1997). So, various dosages and exposure periods were tried out in this investigation to find the optimum level that yield the best results.

MATERIALS AND METHODS

The nauplii of Indian white shrimp *Fenneropenaeus indicus* (from females of 36-38 g average weight) were reared up to zoea 1 stage in the hatchery and used for the experiments. The larvae were stocked in 1 ton fibreglass tanks filled with UV- treated water further filtered down to 1 micron. The larvae stocked at a density of 100/l were treated with immunostimulants either by bath or by feeding immunostimulant mixed feed. The larvae were fed with mixed microalgae (*Chaetoceros muelleri* and *Tetraselmis suecica* at densities $0.7x10^5$ cells/ml and $3x10^4$ cells/ml respectively) and artificial diet 1 CAR for zoea and 2 CD for mysis (4, 8, 12 and 16 mg/l/day for zoea, mysis, early PL (1and 2) and late PL stages respectively). The daily feed was divided into 6 rations. The artificial diets (1 CAR and 2 CD) were from INVE aquaculture, Belgium and have been used successfully in penaeid larviculture (Jones *et al.*, 1987). Larvae were staged according to description given by Silas *et al.* (1978). Daily water exchange rate was 30% for zoea3, 50% for all mysis stages and 100% for postlarval stages.

Immunostimulants

The immunostimulants used in the experiments were (a) yeast glucan, with β-1,3 glucan chemical structure, purchased from Sigma Chemical Co., St.Louis, USA, (b) Vetrigard, a commercial immunostimulant with a blend of 1.3, 1.6 glucan, mannan oligosaccharides and peptidoglycans. The glucan derived from the yeast (*Saccharomyces cerevisiae*) and the mannan oligosaccharides from the bacterium (*Bifidobacterium thermophilum*). Vetrigard is a product of Vetrepharm limited, Hampshire, UK. and (c). Levamisole (levo-isomer of tetramisole), a synthetic immunostimulant, was supplied by Merck KGaA. Darmstadt. Germany. Addition of immunostimulant to rearing water and to artificial diet was tried out as

has been suggested by Skjermo *et al.* (2001). Dosages of immunostimulant were selected based on the trials conducted before the actual experiments.

Immersion experiments

The immunostimulants, yeast glucan, vetregard and levamisole were added to the rearing medium in three different dilutions (0.025mg/ml, 0.05 mg/ml and 0.075 mg/ml). Larvae in each dilution were exposed to the immunostimulants for 1.5, 3 and 4.5 hours during the zoea1, mysis1 and postlarval and 3 stages respectively. In control treatments, the larvae were reared with the same density and quantity feed without immunostimulants. A hapa-like structure was used to carry out the transfer of larvae for the treatment and to return the larvae to immnostimulant-free water after the exposure period.

Oral experiments

The larvae and postlarvae were fed with feed mixed with immunostimulant (using fish oil) at 10, 20 and 30 mg/kg levels. Control tank larvae received immunostimulant-free feed but with fish oil addition. Three different treatment regimes with were tried out, (a) stimulatnt incorporated feed fed only to zoea 1, mysis 1 and PL 1 stages (ZP_1), (b) feed given to zoea 1, 2, mysis 1, 2 and PL 1, 2 stages (ZP_2) and (c) feed administered to all zoeal and mysis stages and postlarvae till PL3 stage (ZP_3).

Survival and growth analysis

The overall survival from Z_1 to PL_5 stage (which were the end product of larval rearing section and was reared later in the postlarval section) from all the treatments were noted down by counting five 1 litre samples from PL_5 tanks. Total length was measured using the standard procedure.

Pathogen challenge test

A virulent pathogenic luminescent *Vibrio harveyi* (PLB3) isolated in association with diseased *F. indicus* postlarvae was used for the experiments.

The Z_3, M_3 and PL_5 stages from the immersion treatments and the similar larval stages from oral treatments were exposed to pathogen challenge test. The bacteria were cultured overnight in tryptic soy broth (TSB with 3% NaCl) at 28°C and centrifuged at 5700 rpm for 7 minutes, washed and resuspended in filter-sterilised seawater. The different larval and postlarval stages from control and those treated with immunostimulant were then challenged by immersion method at a concentration of 4 x 10^6 cfu/ml. Beforehand, the relationship between the optical density at 550 nm wavelength (using a spectrophotometer) and the plate count was established by plating dilutions of a suspension with known values of optical density (at 550 nm) on marine agar plates. The bacterial concentration was based on the preliminary experiments, where the larvae were infected at concentrations ranging from 10^4 to 10^7 cfu/ ml.

The challenge tests with 100/l density larvae and post larvae were conducted for 24 hours and survival after this period was noted down. Challenge tests were conducted in two litre flasks containing 1 litre of sterile filtered seawater. While the zoeae and myses were fed with algae, the myses and postlarvae were fed with *Artemia*. The water temperature was maintained at 29°C and aeration was provided continuously.

No challenge tests were done for Z_3 and M_3 stages from oral experiments under ZP_3 regime. This is because, while, feeding these larval stages with required feed (as per the regime) itself, they would have moulted to next stage. For example Z_3 stage when the feeding course was completed would be moulting or might have moulted to mysis. It is important to note that only Z_3, M_3 and PL_5 were exposed to challenge tests.

Data analysis

The significance of data between the treatments was analysed by single factor Anova. The significance between individual means was found by using Tukey's test for multiple comparisons.

RESULTS

Immersion treatment

The results of the immersion treatments are as given in tables 40 and 41. Among the three stimulants tried for the shrimp larvae and postlarvae, best results were obtained with yeast glucan, followed by vetrigard. Levamisole recorded comparatively poor results.

a. Yeast glucan:

Best over all survival and PL_5 growth, were from the larvae exposed to highest dose (0.075 mg/ml) with maximum exposure period (4.5 hours). The challenge tests with Z_3, M_3 and PL_5 gave best results with the same treatment. Analysis showed that only the highest overall survival value with glucan was significantly higher than the control ($P<0.05$). With PL_5 length, all the treatments recorded values significantly higher than the control ($P<0.05$). With Z_3 challenge tests, all treatments with different dosages recorded higher survival than the control. Only 0.05 and 0.075 mg/ml dose treatments resulted in values significantly higher than control ($P<0.05$). Challenge tests with M_3 resulted in significant improvement of survival in all treatments when compared to control ($P<0.05$). In PL_5, the results showed that with lower concentrations of immunostimulants (0.025 and 0.05 mg/ml) only the 4.5 hour exposure resulted in significant improvement of survival ($P<0.05$). In the 0.075 mg/ml

Table 40. PL_5 survival and PL_5 total length in treatments exposed to three different immunostimulants through immersion at three concentrations for 1.5, 3 and 4.5 h.

Treatment	PL_5 survival (%)				PL_5 total length (mm)			
	Exposure period (h)				Exposure period (h)			
		1.5	3.0	4.5		1.5	3.0	4.5
Control	43.70 ± 2.11				7.28 ± 0.071			
Glucan								
0.025mg/ml		53.67 ± 2.90	51.30 ± 4.37	54.30 ± 4.32		7.48 ±0.031*	7.51 ± 0.034*	7.56 ± 0.066*
0.05mg/ml		51.00 ± 2.61	54.00 ± 4.13	55.70 ± 3.80		7.61 ± 0.047*	7.64 ± 0.082*	7.68 ± 0.042*
0.075mg/ml		56.00 ± 2.77	58.70 ± 3.15	65.20 ± 5.07*		7.71 ± 0.019*	7.74 ± 0.094*	7.78 ± 0.089*
Vetrigard								
0.025mg/ml		53.10 ± 2.76	55.70 ± 2.93	56.11 ± 3.67		7.51 ± 0.033*	7.54 ± 0.078*	7.60 ± 0.125*
0.05mg/ml		60.14 ± 3.11	59.43 ± 2.97	61.73 ± 2.40*		7.58 ± 0.053*	7.67 ± 0.035*	7.78 ± 0.028*
0.075mg/ml		60.40 ± 2.64	56.31 ± 3.28	58.00 ± 4.11		7.62 ± 0.075*	7.53 ± 0.053*	7.39 ± 0.042
Levamisole								
0.025mg/ml		46.10 ± 3.33	44.90 ± 2.66	49.10 ± 4.41		7.33 ± 0.022	7.36 ± 0.044	7.41 ± 0.042
0.05mg/ml		47.33 ± 2.98	49.67 ± 3.80	53.41 ± 3.74		7.36 ± 0.060	7.42 ± 0.026*	7.47 ± 0.034*
0.075mg/ml		49.00 ± 3.46	53.30 ± 4.17	55.23 ± 3.62		7.37 ± 0.042	7.40 ± 0.029	7.41 ± 0.052

* denotes values significantly different from the control ($P<0.05$).

Table.41. Survival (%) of larvae and postlarvae from the immersion treatments following a challenge with *Vibri harveyi* at 4 x 10^5 cfu/ml density.

Treatment	Zoea 3				Mysis 3				Post larva 5			
	Exposure period (h)				Exposure period (h)				Exposure period (hrs)			
		1.5	3.0	4.5		1.5	3.0	4.5		1.5	3.0	4.5
Control	41.00 ± 3.27				47.33 ± 3.68				63.33 ± 2.76			
Glucan												
0.025mg/ml		50.33 ± 3.41	53.00 ± 2.94	53.00 ± 2.94		63.33 ± 2.87*	64.67 ± 4.11*	68.00 ± 3.27*		70.67 ± 1.18	72.00 ± 2.88	75.67 ± 1.52*
0.05mg/ml		58.00 ± 3.27*	61.33 ± 2.99*	63.67 ± 2.49*		66.00 ± 6.16*	67.00 ± 3.35*	68.00 ± 4.03*		72.00 ± 1.36	75.00 ± 1.09	77.00 ± 2.62*
0.075mg/ml		61.00 ± 5.72*	61.00 ± 4.32*	67.33 ± 3.74*		70.33 ± 4.50*	72.33 ± 2.16*	75.33 ± 1.36*		80.67 ± 1.09*	86.00 ± 0.98*.	86.33 ± 2.72*
Vetrigard												
0.025mg/ml		55.00 ± 2.83	59.00 ± 3.56*	60.00 ± 2.94*		62.00 ± 1.63	65.00 ± 2.59*	67.70 ± 2.88*		69.00 ± 3.66	73.33 ± 2.76	75.00 ± 2.88
0.05mg/ml		62.67 ± 3.40*	65.00 ± 4.32*	66.00 ± 4.08*		63.33 ± 2.87*	64.00 ± 3.14*	69.33 ± 2.42*		71.00 ± 4.01	75.33 ± 1.78*	76.67 ± 2.50*
0.075mg/ml		64.00 ± 3.74*	63.00 ± 5.35*	65.33 ± 3.86*		64.00 ± 3.56*	64.70 ± 2.47*	61.00 ± 4.55		67.00 ± 2.94	64.33 ± 3.21	65.00 ± 3.57
Levamisole												
0.025mg/ml		47.33 ± 2.84	51.33 ± 3.68	53.00 ± 4.97		54.00 ± 3.56	57.33 ± 4.71	59.00 ± 2.83		65.67 ± 3.93	68.00 ± 2.68	66.67 ± 2.42
0.05mg/ml		54.67 ± 2.05	54.00 ± 5.10	56.33 ± 3.86		56.00 ± 2.83	54.67 ± 3.09	56.00 ±.4.55		67.33 ± 2.23	61.33 ± 4.65	63.00 ± 4.77
0.075mg/ml		53.33 ± 4.92	50.00 ± 6.68	52.67 ± 4.50		61.67 ± 3.30	60.33 ± 6.69	62.00 ± 5.35		61.00 ± 4.72	64.00 ± 4.90	64.00 ± 3.74

* denotes values significantly different from the control ($P<0.05$).

concentration even lesser exposure (1.5 h) resulted in significant increase in survival ($P<0.05$), compared to respective control.

b. Vetrigard:

The best result for growth and survival was given by 0.05 mg/ml dose for 4.5 hours. Higher dose even with minimum period of exposure resulted in lesser values for all parameters. Similar to glucan here also only the highest over all survival value showed significant ($P<0.05$) increase when compared to control. With PL_5 length, all the treatments except the one with maximum exposure to maximum dose were significantly higher than control ($P<0.05$).

Challenge tests with Z_3 showed that except for the 0.025 mg/ml concentration with 1.5 hour exposure, all other treatments recorded significantly higher survival ($P<0.05$). Though M_3 also showed a similar pattern of results, only difference was that the larvae from tanks with maximum concentration (0.075 mg/l) and exposure period (4.5 h) also failed to show significant increase in survival ($P>0.05$). Interestingly, with PL_5, the survival was significantly higher only for the medium concentration (0.05 mg/l) for the 3 and 4.5 hour exposures. All the exposure to higher concentrations resulted in lesser survival values.

c. Levamisole:

Compared to glucan and vetrigard, the results with growth and survival were poor. In the case of survival, though all the treatments recorded improved survival, there was no significant increase ($P>0.05$). With PL_5 length, significant increase ($P<0.05$) was recorded only with 0.05 mg/ml concentration in 3 and 4.5 hour exposures. Noticeably, the highest PL_5 length recorded was significantly lower ($P<0.05$) than the highest values obtained with glucan

and vetrigard. Challenging of Z_3 and M_3 larvae with pathogenic bacteria though showed improved resistance and survival, the values were not significantly higher than control ($P>0.05$). In the case of PL_5, the improvement in survival, when compared to control, was only marginal, and none of them were significantly higher than control. Best result was with 0.005 mg/ml in the 1.5 hour exposure period. All the concentrations and exposure above this level resulted in decreased survival.

Oral treatment

Results from the oral experiments are presented in tables 42 and 43. Contrary to immersion treatments, vetrigard gave the best results, followed by glucan. Levamisole, as with immersion treatments though registered poor results, the performance was better in this way of administration.

a. Yeast glucan:

The best over all survival was noted in ZP_3 regime with a dose of 20mg/kg immunostimulant incorporated feed, and was the only one with survival value significantly higher than control ($P<0.05$). PL_5 growth was significantly higher than control ($P<0.05$) for all regimes and in all concentrations.

Challenge test with Z_3 showed that ZP_2 regime with 30 mg/kg feed immunostimulant level was the most optimum and only one with significantly higher survival ($P<0.05$). All inclusion levels with ZP_1 regime failed to improve the survival significantly ($P>0.05$). With M_3, all the treatments except ZP_1 regime (with 10 to 20 mg/kg), resulted in significantly higher survival than control ($P<0.05$). In the case of PL_5, survival indicated an increasing trend with increased stimulant inclusion level in ZP_1 regime. With ZP_2 and ZP_3 regime, increase in inclusion level above 20mg/kg level resulted in reduced survival. Best survival was recorded

Table 42. PL_5 survival and PL_5 total length in treatments fed with immunostimulant incorporated feed under different regimes with different feeding level.

Treatments	PL_5 survival (%)				PL_5 total length (mm)			
		ZP_1	ZP_2	ZP_3		ZP_1	ZP_2	ZP_3
Control	42.33 ± 2.20				7.26 ± 0.024			
Glucan								
10mg/kg		48.60 ± 2.70	52.40 ± 4.00	50.90 ± 2.84		7.44 ± 0.038*	7.49 ± 0.041*	7.53 ± 0.045*
20mg/kg		54.20 ± 3.70	58.40 ± 2.70	61.40 ± 3.10*		7.50 ± 0.033*	7.58 ± 0.033*	7.64 ± 0.031*
30mg/kg		57.90 ± 2.30	57.70 ± 3.60	55.33 ± 4.21		7.69 ± 0.043*	7.70 ± 0.022*	7.74 ± 0.045*
Vetrigard								
10mg/kg		49.30 ± 3.20	53.00 ± 3.75	56.70 ± 2.33		7.48 ± 0.025*	7.57 ± 0.036*	7.64 ± 0.027*
20mg/kg		53.67 ± 3.72	62.67 ± 2.12*	64.67 ± 2.14*		7.60 ± 0.031*	7.82 ± 0.033*	7.71 ± 0.030*
30mg/kg		56.10 ± 2.66	54.33 ± 2.80	52.33 ± 2.71		7.61± 0.035*	7.57 ± 0.032*	7.51 ± 0.061*
Levamisole								
10mg/kg		42.70 ± 2.70	46.40 ± 3.60	47.70 ± 3.22		7.37 ± 0.033	7.46 ± 0.065*	7.40 ± 0.050*
20mg/kg		44.20 ± 2.93	49.30 ± 4.28	48.80 ± 3.43		7.40 ± 0.035*	7.50 ± 0.049*	7.30 ± 0.060
30mg/kg		42.00 ± 3.64	56.60 ± 3.80	44.50 ± 3.90		7.33 ± 0.065	7.31 ± 0.102	7.28 ± 0.024

* denotes values significantly different from the control ($P<0.05$).(Anova followed by Tukey's test)

Table 43. Survival (%) of larvae and postlarvae (fed immunostimulant incorporated feed) following a challenge with *Vibrio harveyi* at 4 x 10^5 cfu/ml concentration.

Treatments & stimulant dose	Survival (%)						
	ZOEA 3		MYSIS 3		POSTLARVA 5		
	ZP_1**	ZP_2	ZP_1	ZP_2	ZP_1	ZP_2	ZP_3
Control	38.00 ± 2.94		46.00 ± 6.16		54.67 ± 6.13		
Glucan							
10mg/kg	44.33 ± 4.92	47.00 ± 5.10	56.00 ± 7.87	68.67 ± 4.78*	71.00 ± 6.48	75.00 ± 5.10*	80.00 ± 2.45*
20mg/kg	53.00 ± 7.26	53.33 ± 3.68	60.67 ± 4.50	72.00 ± 4.97*	78.33 ± 5.73*	79.00 ± 3.74*	80.67 ± 3.68*
30mg/kg	55.67 ± 3.68	58.00 ± 1.63*	65.00 ± 4.55	73.00 ± 3.56*	81.00 ± 2.94*	76.00 ± 4.55*	76.70 ± 6.02*
Vetrigard							
10mg/kg	46.00 ± 6.68	51.33 ± 6.02	64.00 ± 2.94	68.33 ± 5.79*	73.00 ± 5.10	73.67 ± 6.60	77.00 ± 5.72*
20mg/kg	54.00 ± 5.35	59.33 ± 5.31*	70.00 ± 5.35*	68.00 ± 4.90*	74.67 ± 4.19	82.67 ± 3.74*	78.00 ± 4.19*
30mg/kg	61.00 ± 4.90*	63.67 ± 3.74*	72.00 ± 2.45*	74.67 ± 4.19*	73.33 ± 4.03	71.00 ± 5.10	68.00 ± 4.24
Levamisole							
10mg/kg	41.00 ± 5.10	47.33 ± 5.44	53.33 ± 2.62	57.00 ± 4.90	67.00 ± 4.97	69.33 ± 6.55	63.00 ± 7.12
20mg/kg	50.00 ± 4.32	47.00 ± 6.38	64.00 ± 4.55	54.67 ± 4.50	64.00 ± 2.83	69.67 ± 2.05	62.00 ± 5.10
30mg/kg	49.33 ± 4.50	48.33 ± 4.64	59.33 ± 3.40	56.00 ± 6.98	67.00 ± 6.53	65.00 ±5.66	60.00 ± 4.08

* denotes values significantly different from the control (P<0.05)

by PL_5 from ZP_1 regime fed 30 mg/kg with almost similar survival from PL_5 of ZP_3 regime receiving 20 mg/kg of immunostimulant.

b. Vetrigard:

The best over all survival and growth was obtained in ZP_3 and ZP_2 regime with 20 mg/kg inclusion level respectively. Higher inclusion level resulted in lesser survival and growth. Interestingly, the survival obtained here was slightly higher than that of glucan (64.67 against 61.4%). Challenge tests with Z_3 proved that 10 mg/kg inclusion level under ZP_1 and ZP_2 regime and 20 mg/kg level under ZP_2 regime did not produce any significant improvement in survival ($P<0.05$). In M_3 except 10 mg level in ZP_1 regime, all other gave significantly higher survival ($P<0.05$). However, with PL_5, 20 mg/kg inclusion was noticed to be optimum, registering best results with all three regimes. Best challenge survival was noted with ZP_2 regime.

c. Levamisole:

With oral experiments also levamisole recorded lower response from larvae, but with slightly better results than the immersion route. The Z_1- PL_5 survival value, compared to control, had increased in all treatments only slightly and did not show any significant improvement ($P>0.05$). With PL_5 the maximum growth was (with ZP_2 regime at 20 mg feeding level) significantly lower than that of respective glucan and vetrigard values. The Z_3, M_3 and PL_5 challenge test, though showed improved resistance, failed to show any significant improvement in survival ($P>0.05$). While, ZP_1 regime with 20mg/kg level was found to be the most optimum for Z_3 and M_3, it was of ZP_2 regime with similar dose for PL_5.

DISCUSSION

Our knowledge on the crustacean immune system is limited and the economical importance of shrimp has prompted the necessity to study their immune components by many authors (Song and Hsieh, 1994; Rodriguez *et al.*, 1995; Lopez *et al.*, 1996; Sung *et al.*, 1998; Destoumieux *et al.*, 2000). The immune system of crustacean is relatively primitive and does not produce antibodies, and their immune responses to resist infections are not specific in nature (Soderhall and Cerenius, 1998). This means that crustaceans, including shrimp, can not be "vaccinated" in the traditional definition. However, there are commercial products that claim to stimulate disease resistance.

The immunostimulants attach to specific receptors on the cell surface of phagocytes and lymphocytes, activates the cell resulting in increased production of enzymes that can destroy pathogens. Immunostimulants may also act by blocking the bacterial adhesion to specific receptors on target cells.

The immunostimulant or its chemical components used in these experiments (β-1.3 / 1.6 glucan, peptidoglycan and levamisole) have been tried before with shrimp juvenile and adult (Song and Hsieh, 1994; Song *et al.*, 1997; Sung *et al.*, 1994, 1998; Supamattaya and Pongmaneerat, 1998; Kumar, 1996; Devaraja *et al.*, 1998; Raa, 2000). Levamisole was tried as immunostimulant for *Macrobrachium rosenbergii* postlarvae by Baruah and Prasad (2001).

Overall results showed that with glucan immersion treatment of larvae yielded best results, while it was with oral administration in the case of vetrigard and levamisole. Analysing the effect of individual compounds on the growth and response to pathogen would help in developing a strategy for their application. Evaluating three immunomodulators

(glucan, levamisole and chitosan) for *F. indicus* juvenile by incorporating them in the feed, Kumar (1996) found that the best growth and survival was given by glucan. In this experiment, with immersion experiments, the overall performance was better for glucan than vetrigard and levamisole. However, in the oral experiments, better results were recorded with vetrigard than glucan. In the oral experiments, the less efficiency of glucan could be due to the fact that crustaceans digest them (Glass and Stark, 1995; Lopez *et al.*, 2003), making it likely that very little biologically active material gets into the animal.

The better performance of vetrigard could be due to its chemical composition including β-1,3 glucan, mannan oligosaccharides and peptidoglycan and possible synergic effect. Pro-phenoloxidase system (pro-PO) may be activated by β-1,3 / 1,6 glucan, peptidoglycans and lipopolysaccharides (Soderhall *et al.*, 1994; Barracco *et al.*, 1991). β-1,3 glucan and peptidoglycan have been recognised as elicitors of the pro-PO system (Yoshida and Ashida, 1986; Soderhall *et al.*, 1990). In its active form, phenol-oxidase (PO) catalyses the oxidation of phenols to semi-quinones and quinones, which due to their high reactivity, will kill microbes. The mannan oligosaccharides are said to directly interfere with the adhesion of bacteria to host cell (vetrigard product literature).

Interestingly, Devaraja *et al.* (1998) reported that mixtures of lipopolysaccharides (LPS) and glucans may work together synergistically. With vetrigard, even if the glucan part of the stimulant was digested, the peptidoglycan and mannan oligosaccharides took care of immune-stimulating function or could be that much glucan was spared and contribute to larval nutrition. In this study, the cumulative effect on immune-boosting was noticed only with oral experiments and again the results were not much different from glucan results. The nutritious role by vetrigard was proved by the better PL_5 growth in the oral experiments and the growth was similar to glucan fed PL_5 in immersion experiments.

Studies with fish and shrimp have proven the growth boosting, survival promoting and immune-stimulating effect of β-glucan. β-1,3 / 1,6 glucan has been administrated through immersion as well as the oral route (Supamattaya and Pongmaneerat, 1998; Sung *et al.*, 1994), but no literature is available comparing the effect of application routes on performance. Injection of β-1,3 / 1,6 glucan into fish resulted in their increased resistance to several bacterial diseases (Robertsen *et al.*, 1990, Samuel *et al.*, 1996) and enhanced efficacy of the vaccine (Rorstad *et al.*, 1993). Immersion of shrimp postlarvae in β-1,3 glucan increased their survival (Supamattaya and Pongmaneerat, 1998). Post larval tiger shrimps showed enhanced growth and resistance to *V. vulnificus* for eighteen days following the administration of beta glucan (Sung *et al*, 1994). β-1,3 / 1,6 glucan enhanced the biological activity of shrimp haemocytes, improved growth, survival and feed conversion efficiency (Sung *et al.*, 1994, 1998; Song and Hsieh, 1994; Song *et al.*, 1997; Chang *et al.*, 1999; Dugger and Jory, 1999). Scholz *et al.* (1999) also reported improved vibriosis resistance in juvenile *L. vannamei* with the use of β-1,3 / 1,6 glucan.

Enhanced bactericidal activity in the haemolymph of *P. monodon* treated with β-glucan, zymosan or *Vibrio* bacterin was reported by Sung *et al.* (1996). Devaraja *et al.* (1998) reported induction of vibriocidal activity in the haemolymph of *P. monodon* fed with a diet containing yeast β-glucan. Felix *et al.* (2004) reported that addition of a commercial immunostimulant to feed enhanced immunity and growth of postlarval stages of *P. monodon.*

Supamattaya *et al.* (2000) used β-glucan (MacroGard) for immunostimulating *P. monodon* juveniles through feed for 11 weeks, and found that supplementation of MacroGard at different levels (0.25, 0.5 and 1 g/kg feed) in diets had no effect on growth, survival and feed conversion. However, resistance to pathogenic bacteria (*V. harveyi*) increased with increasing dose. Such an effect suggested more influence of glucan on the

immune boosting than over all survival was noticed with immersion treatments in this investigation. Though a significant increase in PL_5 survival (with respect to control) was obtained only for the 0.075 mg/ml concentration with 4.5 hour exposure, challenge test survival with Z_3, M_3 and PL_5 made significant increment even for the lower dosages and exposure.

Another interesting fact was that even though all the zoea and mysis stages were exposed or fed with glucan, significant increase in challenge test survival of PL_5 still required higher exposure dose or period. For example, in immersion method, the lower level dosages (0.025mg/ml and 0.05 mg/ml) required 4.5 hour exposure to make significant improvement in challenge test survival of PL_5 (Table 41). Similarly, in oral experiments, 10mg/kg level (glucan incorporated) feed had to be given up to PL_2 or higher dose (20mg/kg) up to PL_1 to achieve significant survival improvement in challenge test with PL_5. So, it can be deduced that continuous application of immunostimulant is required to get better results. Moreover, higher the dosage longer was the resistant period, but within the optimum level. While reporting enhanced resistance of shrimp postlarvae to bacterial infection (*V. vulnificus*) with β-1,3 / 1,6 glucan administration, Raa (2000) suggested that to maintain elevated levels of resistance immunostimulant had to be used on a continuous basis.

Though peptidoglycan was not used separately in this investigation, studies have shown the individual ability of the same as immunostimulant. Boonyaratpalin *et al.* (2000) tried peptidoglycan extracted from *B. thermophilum* in *P. monodon* for five weeks. The results on clearance ability of shrimp against bacteria in the haemolymph showed that the shrimps fed with peptidoglycan supplemented diets were able to eliminate 90% of bacteria whereas the control shrimps could eliminate only 50%. Moreover, shrimps fed with peptidoglycan supplemented diets were more resistant to *V. harveyi* infection than the control group.

Boonyaratpalin *et al.* (1995) noted that peptidoglycan-fed *P. monodon* exhibited higher tolerance to dissolved oxygen, salinity and stress. The positive results obtained with vetrigard in this experiment also could be due to multiple role as mentioned above. Improved resistance of shrimp to virus with peptidoglycan has also been reported (Boonyaratpalin *et al.*, 1995; Itami *et al.*, 1998; Takahasi *et al.*, 1998). As no reports are available, individual assessment of peptidoglycan and mannan oligosaccharides is wanted to understand their effectiveness for shrimp larval immunostimulation.

With levamisole, the results were comparatively poor, suggesting its less suitability for immunostimulation of shrimp larvae when compared to glucan and vetrigard. The results with immersion treatment for all parameters were not significant (except for the PL_5 growth in two treatments). The oral route of administration resulted in four of the treatments with significant increase in postlarval length and survival of Z_3 and M_3 at least in one of the treatments. Such poor results, even with oral treatment of levamisole have been reported for juvenile *F. indicus* (Kumar, 1996). However, levamisole expressed its growth enhancing and immunostimulating effect in the present experiments. With postlarvae of freshwater prawn *Macrobrachium rosenbergii*, Baruah and Prasad (2001) reported that dietary intake of levamisole had no significant impact on the gain of body weight. Meanwhile, the prawns showed an enhanced phenoloxidase activity and increased resistance to pathogenic bacterium, *Pseudomonas fluorescens.*

Challenging the animal with a pathogen after exposing them to immunstimulant has been done to analyse the influence of the stimulant on improving animal's disease resistance (Skjermo *et al.*, 1996; Scholz *et al.*, 1999; Supamattaya *et al.*, 2000). Pathogenicity of *Vibrio* species like *V. alginolyticus, V. parahaemolyticus* and *V. harveyi* on shrimp larvae and

juvenile have been studied by many (Hameed, 1994; Prayitno and Latchford, 1995; Uma, 1995; Abraham and Shanmugam, 1996; Felix and Murugan, 2003).

Prayitno and Latchford (1995) noted that the virulence of bacteria (*V. harveyi*) on shrimp (*P. monodon*) larvae was related to the age of the larvae such that higher stages have more resistance to pathogen. A similar effect was also noticed in the present investigation. However, comparison of results is not advised as differences in the pathogenicity of bacteria could depend on the species tested (Vera *et al.*, 1992) and on the strain characteristics (de la Pena *et al.*, 1993).

It is vital to note that imbalance in the nutrient composition of diet affects the growth and general performance of an animal and most likely the biochemical processes of the immune system. The performance of zoeal stages are much influenced by the nutritional status of preceding naupliar stages which are lecithotrophic. Vitamin C, polyunsaturated fatty acids (PUFA) and vitamin E have been proven to influence disease resistance in fishes (Hardie *et al.*, 1991; Satyabudhy *et al.*, 1989; Waagbo *et al.*, 1993; Erdal *et al.*, 1991). Lopez *et al.* (2003) suggested long-lasting immunological role of a mega dose of vitamin C, when compared to glucan in *Litopenaeus vannamei* juveniles. High levels of long chain fatty acid in the diet of Atlantic salmon resulted in immunosuppressive effect, resulting in reduced survival rates in challenge tests with *V. salmonicida* and *Yersinia ruckeri* (Erdal *et al.*, 1991).

In conclusion, the results of this study showed that all the three immunostimulants had positive effect on the growth, overall survival up to PL_5 and the pathogen resistance of larval stages. Even with the same immunostimulant, the results for the parameters differed based on the dosage and exposure hours. Similarly, the three differed in their efficacy depending on the route of administration, dosage and exposure hours. Following method of

immunostimulant administration, dosages and exposure periods resulted in the overall best survival, growth and disease resistance of larvae and postlarvae in shrimp hatchery.

Glucan – Immersion – 4.5 hours – 0.075 mg/ml concentration

Vetrigard – Oral route – ZP_2 regime – 20 mg/kg level incorporated in the feed

Levamisole – Oral route – ZP_2 regime – 20mg/kg level incorporated in the feed

However, it is recommended that initial trials were made to analyse the influence of nutrition provided to larvae, for example, hatcheries do add vitamin C to rearing water and such actions could reduce the requirement of immunostimulant. Further studies with different treatment regimes and comparison of challenge tests with other pathogens prevalent in the hatchery environment are wanting. Such studies could help to develop a standard application regime for the immunostimulants in the hatchery. Increased growth, survival and disease resistance with the use of stimulants would undoubtedly increase the health of hatchery product, by giving disease resistant postlarvae and thus benefits surpassing the cost incurred.