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†Immunostimulants in the immune response of *Penaeus monodon* (Fabricius)

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Abstract

The effectiveness of three immunostimulants *viz*., vitamin C, levamisole and chitin on the prophenoloxidase (proPO) activity of haemolymph, response to challenge studies and growth performance of juveniles of the tiger shrimp *Penaeus monodon* (Fabricius) was assessed by oral administration of these immunostimulants to the shrimps. The levels of incorporation of these immunostimulants in shrimp feed were vitamin C at 50, 150 and 250 mg /kg; levamisole at 5, 50 and 100 mg/kg and chitin at 50, 100 and 200 mg/kg levels. Juveniles of *P. monodon* were fed with the experimental diets (9 immunostimulants + 1 control) for 6, 10 and 15 days duration under laboratory conditions. Immune enhancement was recorded as increase in proPO activity in haemocytes and plasma of experimental juvenile shrimps. These diets resulted in relatively better growth rate than the control. The challenge studies were carried out for periods of 6, 10 and 15 days duration. For the cumulative effects of proPO activity in haemocytes and plasma, along with growth increment and survival rate, levamisole at 5 mg/kg, followed by chitin at 100 mg/kg and Vitamin C at 150 mg/kg were observed to be the effective doses to enable "immune enhancement" in shrimps. The 'prophenoloxidase assay' standardised in the study proved to be an effective and simple tool for measuring the immune enhancement in penaeid shrimps.

Keywords: Chitin, immunostimulants, immune enhancement, levamisole, proPO activity, vitamin C

Introduction

Shrimp aquaculture has developed rapidly in India with increase in the number of hatcheries and farms since late 1980s. Among crustaceans *Penaeus monodon* is the major species cultured and contributes to a significant portion of the exports accounting for 50% of total shrimp exports. The black tiger shrimp *P. monodon* is the widely adopted species in aquaculture due to its faster growth and adaptability to a wide range of salinity, from freshwater to seawater than the other penaeid species (Ravichandran and Pillai, 2004). Though the shrimp culture sector witnessed a major growth during the last decade, the production is currently plagued by various factors like environment degradation and diseases. This situation has grown multifold with intensification of shrimp farming without proper knowledge of shrimp health particularly on the immunology of penaeids. The causative agents of infectious diseases in shrimp are mainly viruses and bacteria. In shrimps, *Vibrio* sp. contributes to a number of epizootic diseases. Of the vibrios associated with penaeid shrimps, *Vibrio alginolyticus, V. parahaemolyticus, V. harveyi, V. vulnificus* and *V. anguillarum* cause serious setbacks in the shrimp culture system and are responsible for shrimp mortalities either directly or indirectly (Nash *et al*., 1992). Marine invertebrates lack an acquired,

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memory-type immunity, based on T-lymphocyte subsets and clonally derived immunoglobulins. Instead, they rely solely on innate immune mechanisms that include both humoral and cellular responses. The immune system of shrimp is relatively primitive when compared to that of fish. In shrimp, non-specific immune system plays an important role in the defence mechanisms. Azad *et al.* (1995) reported non-specific immune systems such as phagocytosis, encapsulation, nodule formation, cytotoxicity, lectins and prophenoloxidase (proPO), which have an important role in the defence mechanism of shrimps. Immunostimulants increase resistance to infectious diseases, not by increased specific immune responses but by improving the non-specific defence mechanisms. Therefore, in the absence of the memory component the response is likely to be of short duration. Immunostimulants comprise a group of biological and synthetic compounds that enhance the non-specific defence mechanism in animals. Levamisole is an anthelmintic drug which is a synthetic pheyli midazolthiazole, used as an immune enhancer in several fish species (Jeney and Anderson, 1993). Chitin is a polysaccharide, usually obtained from arthropods which is normally incorporated in shrimp diet and has been demonstrated as an immunostimulant by Kawakami *et al.* (1998) in shrimp. Vitamin C is structurally one of the simplest vitamins and fish and crustaceans are incapable of biosynthesis of ascorbic acid and therefore, diet is the only source of Vitamin C (Akiyama *et al.*, 1992). The immune enhancement efficiency of Vitamin C has been reported in the channel catfish, *Ictalurus punctatus* by Duncan and Lovell (1994).

Material and Methods

Postlarvae of *P. monodon* produced from a nearby hatchery were acclimatised to the salinity 30±1 ppt and maintained in the water recirculation system (WRS) in the laboratory. Shrimps of weight 2.28±0.23 g were used in the experiment. The temperature ranged between 28.0 and 31.5°C in the indoor experimental tanks. The test diets were formulated and prepared with a standard basal shrimp diet composition (Chin, 1988). Inclusion levels of immunostimulants in experimental diets are shown in the Table 1. Feeding was *ad libitum* and scheduled twice a day at 0600 and 1800 hrs. The acclimatised seeds were transferred to plastic tubs of 45 litres capacity installed in the WRS. A total of 40 tubs were used for the experiment comprising 2 each for test feed with Vitamin C, levamisole and chitin and 4 for the control feed. For each test feed 4 tubs were maintained with six animals in each tub. The immunostimulants were fed to shrimps for 1, 5 and 10 days respectively, followed by control feed for the next 5 days. At each sample point 6 animals were sacrificed to draw haemolymph. Inoculates from gills, muscle or hepatopancreas of moribund and *V. alginolyticus* infected shrimp were transferred to thiosulphate citrate bile salt sucrose agar (TCBS) plates with 2% sodium chloride, using a sterile loop. After incubation at 37°C for 24 hours, morphologically identical dominant yellow colonies were progressively purified on tryptone soya agar

Table 1. Inclusion levels of immunostimulants in experimental diets

Sl.No.	Immunostimulants incorporated	Source	Inclusion level	Code
1.	Vitamin C	HiMedia, Lab.Ltd, Mumbai	50 mg/kg	VL
			150 mg/kg	VM
			250 mg/kg	VH
2.	Levamisole	Karnataka A&P Ltd, Bangalore	5 mg/kg	LL
			50 mg/kg	LM
			100 mg/kg	LH
3.	Chitin	HiMedia, Lab.Ltd, Mumbai	50 mg/kg	CL
			100 mg/kg	CM
			200 mg/kg	CH

(TSA) with 2% sodium chloride and maintained on TSA slants. Identification of pathogen was carried out by standard biochemical tests as described by Alisna and Blanch (1994). To determine the LD_{50} value, juveniles of *P. monodon* (2.28 ± 0.23 g), were maintained (six numbers) in tubs with 25 litres seawater (salinity 30 ± 1 ppt). The pathogenicity test was carried out as described by Vera *et al.* (1992). The shrimp juveniles were fed with immunostimulants incorporated diets for 1, 5 and 10 days respectively. On the 7th, 11th and 16th days of each experiment the experimental shrimps (including control) were challenged with a pathogenic strain of *V. alginolyticus.* Bacterial cell counts approximating to that of LD_{50} values were injected into the experimental animals. Parallel controls with no immunostimulants treatment and saline (2% sodium chloride) control were also maintained. The mortality pattern was observed for a period of 5 days after challenging. The process of oxidation of prophenoloxidase to phenoloxidase in the presence of active serine protease in the granules of haemocytes can be measured using ELISA Reader in the prophenoloxidase assay (Vargas-Albores *et al.*, 1996; Devaraja *et al.*, 1998). ProPO assay was carried out in the haemolymph (haemocytes and plasma separately) drawn from the shrimps. Haemolymph of 500 µl was centrifuged at 700 g for 5 minutes and the pellet was suspended in 200 µl of cacodylate buffer which was the source for supplying calcium. The cell suspension was incubated with an equal volume of zymosan for 60 minutes. The mixture was centrifuged at 700 g for 5 minutes and the supernatant was placed in micro-titration plate wells and 25 µl of L-DOPA was added. Cacodylate buffer of 300 µl was then added and the absorbance was measured at 490 nm in an ELISA Reader (Lab Systems., Finland) for 10, 20, 30 and 60 minutes. ProPO activity was expressed as the changes in absorbance min^{-1}, mg $protein^{-1}$. Triplicates were prepared for each sample from each of the treatments. ProPO activity for the plasma was measured individually for each sample. Protein concentration of haemolymph was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as the standard protein and OD_{660} of the samples were measured by using a spectrophotometer.

Results

In 6 days, the chitin incorporated diets showed relatively better growth rate than the other two

Table 2. Effect of immunostimulants on immune response in *P. monodon*: bacterial challenge with *Vibrio alginolyticus* (inclusion dose: $1.5x10^5$ cfu/shrimp)

Sl. No.	Treatment	Mean mortality rate (%) for 6 days treatment	Mean mortality rate (%) for 10 days treatment	Mean mortality rate (%) for 15 days treatment
1.	VL	25.01 ± 08.34	41.68 ± 08.34	25.01 ± 08.34
2.	VM	41.68 ± 08.34	33.34 ± 00.00	25.01 ± 08.34
3.	VH	25.01 ± 08.34	25.01 ± 08.34	41.68 ± 08.34
4.	LL	25.01 ± 08.34	16.67 ± 00.00	16.67 ± 00.00
5.	LM	33.34 ± 00.00	25.01 ± 08.34	41.68 ± 08.34
6.	LH	41.68 ± 08.34	25.01 ± 08.34	16.67 ± 00.00
7.	CL	33.34 ± 00.00	25.01 ± 08.34	41.68 ± 08.34
8.	CM	41.68 ± 08.34	16.67 ± 00.00	16.67 ± 00.00
9.	CH	41.68 ± 08.34	41.68 ± 08.34	25.01 ± 08.34
10.	C	50.00 ± 00.00	50.00 ± 00.00	50.00 ± 00.00

immunostimulants. Chitin at higher dose (200 mg/kg) recorded the highest growth rate. Levamisole incorporated diet recorded growth rate next to chitin, at 100 mg/kg concentration. Vitamin C at 150 mg/kg level also recorded better performance in growth than that of the control. In 10 days duration study, levamisole-based diets recorded improved growth rate at 50 mg/kg, followed by 200 mg/kg level of chitin. Among the different levels of Vitamin C incorporation tried, 150 mg/kg level recorded the maximum increment. In 15 days duration experiment chitin at the moderate dose of 100 mg/kg showed relatively better growth rate followed by Vitamin C (150 mg/kg) and levamisole (50 mg/kg). Statistically there was no significant difference ($p>0.05$) between the mean values of specific growth rate for immunostimulant incorporated diets and control diets. However, between the treatment durations a highly significant ($p < 0.01$) relationship was noticed.

Shrimps challenged with doses of $1.3x10^7$ cfu/shrimp and $1.3 x10^6$ cfu/shrimp showed 100% mortality within 120 h. At a challenge dose of $1.3x10^5$ cfu/shrimp, 50% mortality was observed and it was considered as the LD_{50} value. Mortality of shrimps did not occur at the injection dose of $1.3 x10^4$ cfu/shrimp, $1.3x10^3$ cfu/shrimp, saline and uninjected controls. The injected shrimps showed lethargy, erratic swimming, opaqueness [starting from the point of injection (*i.e.* between 4^{th} and 5^{th} abdominal segments) and gradually spreading towards the anterior portion of the abdominal segments] and dorsal flexure in moribund shrimp. Challenging the juvenile shrimps, *P. monodon* with *V. alginolyticus* approximating to that of LD_{50} level ($1.5x10^5$ cfu/shrimp) resulted in different levels of mortality for different concentrations of immunostimulant incorporation. In 6 days treatment, shrimps fed with chitin exhibited lower mortality rate (33.34%) at concentration level of 50 mg/kg. Higher mortality rates (41.68 ± 8.34%) were observed in shrimps fed with test diets containing chitin at 100 mg/kg and 200 mg/kg (41.68 ± 8.34%). In 10 days treatment, the chitin fed shrimps showed lower mortality rate (16.67%) at incorporation level of 100 mg/kg, whereas relatively higher rates of mortalities (25.01 ± 8.34 and 41.68 ± 8.34%) were observed at incorporation levels of 50 and 200 mg/kg respectively (Table 2). However, all the inclusion levels of the above immunostimulants showed relatively better survivals than the control. The results revealed no significant difference between the mean mortality rates of different treatments and treatment durations (Table 3).

The proPO activity of haemolymph separately estimated for haemocytes and plasma are furnished in Table 4. Among the three immunostimulants, vitamin C demonstrated a relatively better immune-enhancement (haemocyte based proPO activity) to the control. Among them, 50 mg/kg recorded the highest proPO value for the 6 days treatment. For 10 days treatment, 250 mg/kg showed the highest activity and 150 mg/kg recorded the highest value among the 15 days treatment. In levamisole test diet for 6 and 10 days treatments 5 mg/kg level recorded the maximum proPo activity. The two-way ANOVA (Table 5) revealed that the haemocyte-based proPO activity recorded significant ($p < 0.01$) increment among the treatments. With respect to increase in duration also the proPO activity recorded significant increment at 1% level ($p < 0.01$). The combined effect among the treatments and control recorded significant increment at 5% level. Plasma based

Table 3. Analysis of variance (ANOVA) for immune response against *V. alginolyticus* challenge in *P. monodon* juveniles

Source of Variation	SS	df	MS	F	P-value	F crit
Between groups	226.9815	2	113.4907	0.940731	0.402765	3.354131
Within groups	3257.306	27	120.641			
Total	3484.288	29				

Table 4. Haemocytes based proPO activity in *P. monodon*

Sl. No.	Prophenoloxidase activity (μ/min/mg protein×10 $^{-5}$)			
	Treatments	6 days	10 days	15 days
1.	Control (C)	0.110 ± 0.007	0.108 ± .0.011	0.109 ± 0.009
2.	Vitamin C			
	50 mg/kg (VL)	0.216 ± 0.027	0.205 ± 0.010	0.475 ± 0.013
	150mg/kg(VM)	0.206 ± 0.014	0.313 ± 0.018	0.528 ± 0.028
	250mg/kg (VH	0.163 ± 0.019	0.355 ± 0.005	0.115 ± 0.028
3.	Levamisole			
	5 mg/kg (LL)	0.163 ± 0.020	0.417 ± 0.026	0.236 ± 0.012
	50 mg/kg (LM)	0.136 ± 0.013	0.358 ± 0.016	0.114 ± 0.016
	100 mg/kg (LH)	0.103 ± 0.019	0.397 ± 0.016	0.239 ± 0.009
4.	Chitin			
	50 mg/ kg (CL)	0.137 ± 0.006	0.348 ± 0.013	0.132 ± 0.006
	100 mg/kg(CM)	0.127 ± 0.014	0.397 ± 0.041	0.237 ± 0.000
	200 mg/kg(CH)	0.117 ± 0.013	0.265 ± 0.025	0.168 ± 0.004

proPO activity revealed that the vitamin C recorded the highest activity at 150 mg/kg level for 15 days treatment and levamisole at 100 mg/kg level for the same duration (Table 6). Chitin, however recorded the peak proPO activity at 100 mg/kg level for 10 days treatment. The plasma based proPO activity (Table 7) did not show any significant difference among the combined treatments and durations. However, significant difference between the treatments was observed at 1% level ($p < 0.1$) and among durations at 5% level ($p < 0.05$).

Discussion

Immunostimulants can be defined as a chemical, drug, stressor or action that elevates the non-specific defense mechanism or the specific immune response (Anderson, 1992; Siwicki *et al*., 1994; Sakai, 1999). The crustacean immune system is heavily dependent on non-specific factors and the specific immune system that is seen in vertebrates seems to be absent in crustaceans (Karunasagar and Karunasagar, 1999). Though invertebrates do not have immunoglobulins they are capable of recognising and destroying the invading microorganisms or parasites (Vargas - Albores *et al*., 1996). Shrimps largely depend on the non-specific defense mechanism to protect themselves against any invasions. In such a perspective studies on the value of cellular and humoral parameters as indicators of shrimp condition are being carried out, with the intention of developing criteria for sanitary surveys, immunostimulation studies and selection programs for shrimp with high

Table 5. Two-way ANOVA for the treatments and control (Haemocytes-proPO)

Co-efficient of Variance	SS	df	MS	F	P-value	F crit
Samples	0.152324	3	0.050775	8.513711	0.0005	3.008786
Columns	0.117792	2	9.876045	9.876045	0.000742	3.402832
Interaction	0.10871	6	0.018113	3.038013	0.023527	2.508187
Within	0.143133	24	0.005964			
Total	0.521965	35				

Table 6. Plasma based proPO activity in *P. monodon*

Sl. No.	Prophenoloxidase activity (μ/min/mg protein×10 $^{-5}$)			
	Treatments	6 days	10 days	15 days
1.	Control	0.140 ± 0.007	0.140 ± .0.006	0.145 ± 0.001
2.	Vitamin C (C)			
	50 mg/kg (VL)	0.213 ± 0.027	0.172 ± 0.020	0.317 ± 0.015
	150mg/kg(VM)	0.147 ± 0.059	0.192 ± 0.023	0.365 ± 0.041
	250mg/kg (VH	0.208 ± 0.021	0.148 ± 0.006	0.149 ± 0.013
3.	Levamisole			
	5 mg/kg (LL)	0.167 ± 0.049	0.196 ± 0.027	0.245 ± 0.160
	50 mg/kg (LM)	0.138 ± 0.014	0.139 ± 0.028	0.175 ± 0.067
	100 mg/kg (LH)	0.141 ± 0.014	0.188 ± 0.016	0.314 ± 0.015
4.	Chitin			
	50 mg/ kg (CL)	0.139 ± 0.000	0.138 ± 0.000	0.143 ± 0.019
	100 mg/kg(CM)	0.143 ± 0.006	0.220 ± 0.000	0.147 ± 0.030
	200 mg/kg(CH)	0.141 ± 0.000	0.141 ± 0.000	0.144 ± 0.009

resistance to pathogens. Several quantitative, fast and easy procedures are being adapted to evaluate the expression of the immune response of shrimp. The proPO system is one of the main defenses, functioning as a non-self recognition system in crustaceans (Johansson and Soderhall, 1989; Soderhall and Cerenius, 1992). Haemocytes and plasma of shrimp haemolymph have been studied for proPO activity by several authors (Sung *et al.*, 1996; Perazzolo and Barracco, 1997; Sritunyalucksana *et al.*, 1999).

Siwicki (1989) reported that carps administered with levamisole by oral treatment recorded, enhanced phagocytic activity and myeloperoxidase activity in neutrophils, increased leucocyte numbers and serum lysozyme levels. In the present study levamisole treated *P. monodon* juveniles recorded the lowest mortality rates *viz.*, 25.01%, 16.67% and 16.67% in 6, 10 and 15 days treatments respectively at the low dose treatment of 5 mg/kg (Table 2). However, at higher doses (50mg/kg and 100mg/kg) increased mortality rates were observed. The above results could be compared to that of the works carried out in *Fenneropenaeus indicus* by Pradeepkumar (1996). *V. alginolyticus* injected into the levamisole treated *F. indicus* juveniles at the dose of 5 mg/kg exhibited the lowest mortality rate (33.31%) and at high concentrations (25 mg/kg and 55 mg/kg) showed high mortality rate (41.66 and 66.66%) in 30 days study. Sung *et al*. (1996) recorded proPO activity in shrimp haemocytes treated by immersion with the vibrio bacterin and glucan. Devaraja *et al*. (1998) reported that immune enhancement through oral administration in *P. monodon* yielded positive

Table 7. Two-way ANOVA for the treatments and control (Plasma-proPO)

Source of Variance	SS	df	MS	F	P-value	F crit
Samples	0.029609	3	0.00987	5.170295	0.006731	3.008766
Columns	0.015009	2	0.007955	4.167064	0.027964	3.402832
Interaction	0.019427	6	0.003238	1.696182	0.165203	2.508187
Within	0.045815	24	0.001909			
Total	0.110761	35				

results in proPO assays. Baruah and Pani (2001) recorded that levamisole incorporated in *Macrobrachium rosenbergii* showed enhanced proPO activity. In the present study immunostimulants were administered orally. A lower dose of 5 mg/kg level of levamisole recorded the highest proPO activity for 6 and 10 days treatment in haemocytes (Table 4). With respect to plasma also it showed the highest proPO activity for the same treatments with same concentration of *leavamisole* (Table 6). Pradeepkumar (1996) reported maximum survival and growth rate at 5 mg/kg levamisole incorporation level in *F. indicus*. The higher dose of 100mg/kg recorded a lower proPO activity both in haemocytes and plasma. Anderson (1992) suggested that higher doses of immunomodulators would suppress defense mechanism in aquatic animals. The works carried out by Kajita *et al.* (1990) in *Oncorhynchus mykiss* with low dose of 5 mg/kg also recorded an increased protection against *V. anguillarum.* Karunasagar *et al*. (1996a) reported the effect of heat killed *V. harveyi* and yeast β-1, 3 glucan and suggested that treatment with either of them would induce the immune response, assessed by vibriocidal activity in haemolymph and haemocytes, proPO activity in haemolymph and haemocytes and the generation of reactive oxygen species in haemocytes. Karunasagar *et al*. (1996b) reported that in grow out systems, regular treatment with immunostimulants resulted in the survival of over 85% in a farm infected with white spot syndrome virus. At the lowest incorporation level (5 mg/kg levamisole), though proPO activity was recorded the maximum, the growth rate was relatively less than that of the higher levamisole concentration (*i.e*. 100 mg/kg). However, the growth rate of test animals fed with 5 mg/kg of levamisole was better than the control.

Chitin at 0.8% level in the diet of *F. indicus* showed a better growth (337.8%) than the control (269.69%) (Vaitheeswaran and Ali, 1986; Fox, 1993). Further, the low molecular weight of chitin can enhance the humoral and cellular immune response in both specific and non-specific system. In the present study a dose of 200 mg/kg of chitin recorded the mean mortality rates *viz*. 41.68%, 41.68% and 25.01% respectively for the treatment period of 6, 10 & 15 days (Table 2) when *P. monodon* juveniles were injected with *V. alginolyticus*. A dose of 100 mg/kg showed the lowest mean mortality rates 41.68%, 16.67% and 16.67% respectively for the same duration. Pradeepkumar (1996) indicated that a dose of 2.5% of chitosan caused mortality of 33.33% in all the treatment durations. Siwicki *et al*. (1994) recorded that the administration of chitosan through injection and orally in the brooktrout increased protection against *Aeromonas salmoncida*. Rainbow trout treated with chitosan by injection or immersion showed increased levels in immunological parameters in the blood such as NBT, potential killing activity, myeloperoxidase and total immunoglobulin concentration (Anderson and Jeney, 1992). Yellowtail injected with chitin alone showed increased protection against *Pleuronectes platessa* challenge, which sustained until 45 days after treatment (Kawakami *et al*., 1998). Sahoo and Mukherjee (1999) reported that improved immunity was observed in healthy and cortisol treated rohu. Anderson and Siwicki (1994) reported that intraperitoneal injection and immersion of chitosan resulted in a higher survival rate than the control in the brooktrout challenged with *A. salmonicida.* In rainbow trout, Sakai *et al*. (1992) investigated the effects of chitin as an immunostimulant and found that intraperitoneal injection elevated the phagocytic activity and chemiluminescent response of leucocytes. In all these studies, only lower doses of chitin or chitosan were used for the immune enhancement. Hence, lower doses of chitin were planned for the present study. In the present study, a dose of 100 mg/kg chitin showed the highest proPO activity in haemocytes in 10 days (Table 4). In plasma the increased proPO activity was also recorded at the same dose for all the treatment durations. However, the other concentrations did not enhance the proPO activity in plasma.

Li and Lovell (1985) demonstrated that channel catfish which were fed diets deficient in ascorbic acid had an impaired antibody response, complement activity and phagocytic engulfment. Tacon and Kurmaly (1996) reported that a dietary vitamin C level lower than 100 mg/kg would avoid deficiencies and ensures maximum growth. In the present study three doses of vitamin C *viz*., 50 mg/kg, 150 mg/

kg and 250 mg/kg were attempted, and the dose of 150 mg/kg of vitamin C showed the maximum activity of proPo at 15 days (Table 4). Comparatively low mortality (25.01%) was observed for the immune response of the juveniles of *P. monodon* to the Vitamin C treatment during the experiment (Table 2). Similar work carried out by Raju (1998) and Hsu and Shaiu (1998) with vitamin C, with a combination of folic acid for a period of 30 days recorded the lowest mortality (33.33%) and improved growth rate at lower concentration of Vitamin C + folic acid. Plasma based proPo activity in *P. monodon* juveniles registered higher levels (from 0.147µ to 0.365µ min/mg protein x 10^{-5}) for the period of 10 days (Table 6) at 150 mg/kg vitamin C treatment. Durve and Lovell (1982) showed that though Vitamin C at 30 mg/kg of diet was sufficient for normal growth, resistance to infection in rainbow trout was achieved at 150 mg/kg of diet. In conclusion, it is observed that three cost-effective immunostimulants vitamin C, levamisole and chitin can be incorporated in shrimp feed at appropriate doses for attaining immune enhancement in shrimps.

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