E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2019; 7(4): 129-133

www.fisheriesjournal.com
Received: 26-05-2019
Accepted: 28-06-2019

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Effects of chitosan on growth, immune responses and survival of juvenile tiger shrimp (*Penaeus monodon* Fabricius, 1798)

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Abstract
Recently the use of chitosan as immunostimulants in aquaculture has been given attention. Four diets (T_1: Basal diet; T_2: 2% extracted chitosan (EC); T_3: 2% commercial chitosan (CC) and T_4: 4% CC) were used to study the effects of chitosan as a growth stimulator and its subsequent impact on immune response in juvenile tiger shrimp. Each diet was randomly assigned in triplicate groups of 60 Juveniles (0.1g ± 0.01) and distributed in 12 glass tanks of 50L. After 42 days of the feeding trial, shrimp were faced with a challenge test of white spot syndrome virus for 6 days. Final weight (FW), percent weight gain (PWG) and specific growth rate (SGR) of shrimp fed diets with chitosan are significantly higher than those of shrimp fed diet T_1 (0% chitosan) (P<0.05). Nevertheless, FW, PWG and SGR of shrimp fed 2% CC are significantly higher than those of shrimp fed 4% CC (P<0.05). No significant difference was recorded in FW, PWG & SGR of shrimp fed T_2 (2% EC) and T_3 (2% CC) (P>0.05). Also, no significant difference was recorded in survival of shrimp during the feeding trial and challenge test (P>0.05). The study revealed that chitosan would be a growth promoter and/or immunostimulant in juvenile tiger shrimp diets with the optimum level of 2%. Considering cost, the locally extracted chitosan is affordable and beneficial for shrimp diets compared to CC.

Keywords: Juvenile tiger shrimp, *Penaeus monodon*, chitosan, growth and immunostimulants

1. Introduction
Sri Lanka is a country involving in large-scale intensive shrimp farming. Most of the shrimp products are export-oriented as a source of income for the local shrimp farmers, revenue for the Sri Lankan government, which have contributed to the economic growth and development of the country. Though, shrimp farming is predominated in brackish waters of Sri Lanka. The Food and Agriculture Organization (FAO) [8] mentioned that shrimp aquaculture contributes 20-25% to the total aquaculture production of Sri Lanka. Moreover, brackish water aquaculture of Sri Lanka in 2014 was 5169 Mt while total aquaculture production was 34211 Mt [8]. Interestingly, the National aquaculture development authority of Sri Lanka (NAQDA) [16] reported that shrimp aquaculture production in the same year (2014) was 5150 Mt. However, according to FAO statistics [8], the brackish water aquaculture of Sri Lanka in 2015 was 7140 Mt while total aquaculture production was 31277 Mt. Meanwhile, NAQDA of Sri Lanka illustrated that shrimp aquaculture production in the same year (2015) was 7090 Mt. Additionally; increasing attention is paid for intensive farming of fish and Shrimp in order to fulfill the decline of natural fish and shrimp population.

Bir *et al.* [5] reported that white spot syndrome virus (WSSV) is the main risk in shrimp production in recent years, especially in Asian countries and it has decreases shrimp production drastically and leads to a substantial economic loss. They also stated that WSSV is a pathogen caused by a viral infection in cultured Penaeid shrimp. Witteveldt *et al.* [23] stated that the disease is transferable and killing shrimps quickly and no proper treatment for this viral disease.

Chitosan which produced by partial deacetylation of chitin is an amino polysaccharide. Chitin is prepared by processing shrimp waste (shell) and it is the second most abundant natural polymer after cellulose [19, 7, 10, 18]. High content of nitrogen (6.89%) in both chitin and chitosan motivates for commercial utilization [19].

Supplementation of nun-nutritional immunostimulants into diets was recognized as an effective practice for enhancing immunocompetence and disease resistance of various aquatic species [17]. Yogeeswaran *et al*. [24] found that the use of immunostimulants with vaccines helped to boost the immune system against WSSV. Similarly, Sakai [20] mentioned that immunostimulants help to minimize aquaculture losses not by all diseases but effective against many diseases. Mastan [15] explained both chitin and chitosan as short term affective non-specific immunostimulators. Chitosan is accepted by fish farmers as an immunostimulant [20], used as an immunostimulant to protect from bacterial disease in fish [6], and exerted an immunostimulatory effect on common carp [10]. However, Ledger *et al*. [14] mentioned fish stress as the critical factor influencing the immune systems of fish in intensive farming. According to the review of Alishahi & Aider [2], chitosan is a potential alternative which can be used as a precautionary source to address fish stress and similar problems. The benefits were observed using dietary supplementation of chitosan in healthy and cortisol treated *Labeo rohita* [15], juvenile Seabass (*Dicentrarchus labrax*) [26] and chitosan and its' nanoparticles in African catfish *(Clarias gariepinus)* [22]. Presently, no published information is available on the use of chitosan as a feed additive for growth or immune responses against white spot syndrome virus or both on *Penaeus monodon*. Therefore, this study was conducted to investigate the effects of Chitosan on growth performances and immunostimulation against white spot syndrome virus of *Penaeus monodon*.

2. Materials and Methods

2.1 Experimental site

A hatchery in a sound environment for the experiment which accomplished with clean, filtered and treated seawater, was selected around Ambakandawila, Chilaw, Sri Lanka.

2.2 Extraction of chitosan

Shrimp shells (Alpex® marine shrimp processing factory, Alaknanda, Wattala, Sri Lanka) were cleaned and washed with running water to remove any remaining muscles and membranes and then dried at 60° C for 2 days continuously. Fragile chitin shells were crushed to make a powder using a motor and pestle. The chitin powder was deproteinized by boiling in 4% NaOH (w/v) for 2hr. Then chitin was washed repeatedly with distilled water until pH dropped to neutral and colourless. Demineralization of the deproteinized chitin was done by boiling it in 4% HCl solution (v/v). The boiling was stopped when there was no bubble detected in excess HCl. Chitin was washed thoroughly in distilled water and dried at 70° C for 5 hr. Finally, chitin was boiled for 12 hr in 40% NaOH solution (w/v) for deacetylation and washed with distilled water and dried; and ground by an electric blender (Sisil blender, Master blend, Singer Sri Lanka) to appear as a fine powder.

2.3 Experimental feeds

CM fish meal® (CMFM), soybean meal and meat and bone meal were used as protein sources. Fish oil was used for the lipid source. Maize, wheat flour and rice polish were used as starch sources and also binders. Coconut meal (coconut poonac) used as an attractant. DL-Methionine and L-Lysine were acted as supplements. Four diets (T_1: Basal diet; T_2: included 2% extracted chitosan; T_3: included 2% imported commercial chitosan and T_4: included 4% imported commercial chitosan) were formulated. Firstly, soya meal, meat and bone meal, maize, coconut meal and, rice polish were grounded by a hammer mill (model: three-phase cap acitorstart motor, China) and passed through a 0.5mm sieve. Then processed dry/ macro ingredients were mixed thoroughly in a planetary mixer (model: 40L multipurpose planetary mixer, China) then micro ingredients were added to the mixer. Finally, fish oil and 20% water were added to appear moist mixture and it passed through an extruder (Model: 50C, Fish feed Extruder, Henan Vic machinery co. Ltd., China) to create 2mm pellets. After drying prepared pellets, it were crumbled and sieved with a 1mm mesh; then stored at – 20ºC until commence of the feed trial.

Table 1: Experimental feed compositions and proximate composition (Means ± SE)

2.4 Experimental design and feeding trial

Recirculatory tank system was used in the trial with 12 glass tanks of 50L. Each feed randomly assigned to three replicates of 60 Juveniles (0.1g±0.01). Shrimp were acclimated to the tank system and experimental feeds for one week. The feeding trial was conducted for 42 days. The system was maintained at 10h dark period and 14h light period. Dissolved oxygen (DO) level and salinity were maintained at 6.5±0.4mg/ L and 30±1 ppt, respectively. The water quality parameters like ammonia nitrogen (NH_4-N), nitrite (NO_2-N) and pH were maintained at 0.04±0.01mg/L, 0.1±0.06mg /L and 7.89±0.11, respectively. The water exchange was done thrice per week and the recirculatory system was run 2h in a day with the flow rate of 3L/ min. Feeding was preceded thrice (8:00, 11.30 and 4:00h) per day. Growth data were collected biweekly. After termination of the feeding trial, the challenge test was initiated.

2.5 Challenge test

Two WSSV infected brooder shrimp were identified by external disease characteristics and followed by a PCR test to confirm the infection. Thereafter, infected brooder shrimp were kept in a tub of 25L until mortalities are recorded. After 10 days, both shrimp died and, the water in their tank which assumed as WSSV contaminated water was equally distributed in 12 tanks (2L/ tank). Feeding was continued and the mortalities of experimental shrimp were recorded for 6 days.

2.6 Sample analysis

Proximate composition analysis of feed ingredients and experimental feeds were performed by the standard method of AOAC [3] in the department of animal science, Faculty of Agriculture, University of Peradeniya and laboratory of Institute of post-harvest technology (IPHT), National Aquatic Resources research and development agency (NARA), Colombo 15.

2.7 Statistical Analysis

One-way analysis of variance (ANOVA) test was used to analyze calculated values. The least significant difference (LSD) was used to find the treatment effect when significant differences were found at a significance level of 5% ($P<0.05$) using IBM SPSS statistics 22.

3. Results

3.1 Experimental diets

Table 1 shows no significant difference according to the

proximate analysis of diets and all diets were isonitrogenous (CP-41.35 ± 0.34 %) and isolipidic (4.81 ± 0.42 %). During 42 days of feeding trials, all experimental diets were well accepted by juvenile shrimp.

3.2 Growth performances

The results on the effects of chitosan on growth and survival of juvenile tiger shrimp (*Penaeus monodon* are presented in Table 02. Final weight (FW), percent weight gain (PWG) and specific growth rate (SGR) of shrimp fed T_2,T_3 and T_4 are significantly higher than those of shrimp fed diet T_1 (0% chitosan) ($P< 0.05$). Nevertheless, the FW, PWG and SGR of shrimp fed T_3 are significantly higher than those of shrimp fed diet T_4 ($P< 0.05$). However, no significant difference was recorded in FW, PWG and SGR of shrimp fed T_2 and T_3 ($P>0.05$). Also, no significant difference was recorded in survival during the feeding trial of shrimp fed four diets ($P>0.05$). However, supplementation of chitosan on final weight of juvenile shrimp shows significantly better results than control diet ($P>0.05$). Notwithstanding, significantly similar effects on final weight show with 2% whether commercial chitosan or extracted chitosan ($P< 0.05$) (Figure 01).

3.2 Challenge test

The results on the effects of chitosan on survival against WSSV of juvenile tiger shrimp (*Penaeus monodon* are presented in Table 02. But, no significant difference was recorded in survival during challenge test of shrimp fed four diets ($P>0.05$).

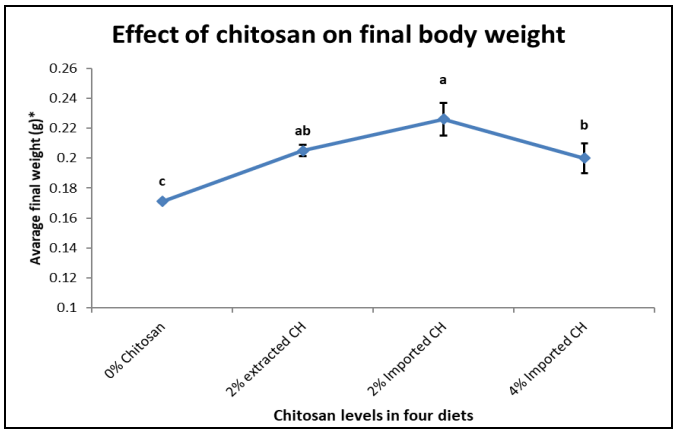
Table 02: Growth performances of juvenile tiger shrimps fed four different diets (Means ± SE) [1].

Figure 01: Effects of chitosan on the final body weight of shrimp fed four different diets.

4. Discussion

Benefits of using chitosan in juvenile tiger shrimp diets were observed in this study. Our study revealed that use of 2% high quality, commercial chitosan and 2% extracted chitosan in juvenile shrimp diets shows a similar effect on growth performances. The price of 1kg high quality commercial chitosan seems to be very expensive (approximately; 250 USD/kg). Therefore, the addition of 2% aforementioned commercial chitosan into juvenile shrimp diet is not practical and it leads to increase the feed cost. Chitosan, which was extracted for our study cost around 10-15 USD. Therefore, if we consider the cost and benefits on the use of the aforementioned commercial chitosan, it is no longer sustainable. Though, the addition of chitosan, which can be synthesized by the above extraction method into tiger shrimp diets, is viable and feasible for optimizing the growth of tiger shrimp (*Penaeus monodon*). Meanwhile, shrimp fed 2% of commercial chitosan diets showed better performance than shrimp fed 4% commercial chitosan diets. It revealed that the optimum supplement levels of chitosan in juvenile tiger shrimp diets should be 2%. Similarly, the effects of chitosan in juvenile Seabass (*Dicentrarchus labrax*) diets were observed by Zaki *et al.* [25] and 1% dietary chitosan showed better growth performances and survival. In contrast, 5% chitosan and 5% chitosan nanoparticle diets showed significantly better growth performances and survival than the control diet for African catfish [22]. Contradictorily tilapia (*Oreochromis Niloticus* x *O. aureus*) growth was depressed by both chitin and chitosan supplementation [21].

During this feeding trial and challenge test, no significant difference was observed in survival ($p< 0.05$). Nevertheless, two studies of Harikrishnan *et al.* [11, 12] reported that the addition of 1% chitin or chitosan in kelp grouper, *Epinephelus bruneus* diets improved immune response against *Philasterides dicentrarchi* and *Vibrio alginolyticus* infections. Similarly the addition of 0.1% *Bacillus subtilis* and 0.6% chitosan in cobia, *Rachycentron canadum* diets improved growth, immune response and disease resistance [9]. Moreover, a supplement of 0.75% chitin increased immune response and disease resistance of *Macrobrachium rosenbergii* [13]. Furthermore, Askariyan *et al.* [4] found that dietary chitin (5%) changes the proportion of gut microbiota and suppress non beneficial gut microbiota to the growth of Atlantic salmon. By the study of Abu-elala [1] again confirmed the commercial application of chitosan as an effective immunostimulant and bio-remediating agent in aquaculture.



*The mean values with different superscript letters are significantly different at $p<0.05$.

Fig 1: Effects of Chitosan on final body weight of shrimp fed four different diets.

Table 1: Experimental feed compositions (%) and proximate composition (Means ± SE)

Ingredient	T_1	T_2	T_3	T_4
CMFM® [1]	33	33	33	33
Extracted Chitosan [2]	0	2	0	0
Imported commercial chitosan [3]	0	0	2	4
Soya bean meal [4]	29	29	29	29
Meat and bone meal [4]	10	10	10	10
Maize [4]	13	13	13	13
Coconut meal [4]	2	2	2	2
Rice polish [4]	4.5	4.5	4.5	4.5
Wheat flour [5]	5	3	3	1
Fish oil [4]	1	1	1	1
Vitamin-mineral premix [6]	2	2	2	2
DL-Methionine [4]	0.3	0.3	0.3	0.3

L lysine [4]	0.2	0.2	0.2	0.2
Proximate composition (DM basis)				
Moisture	7.38±0.15	7.37±0.10	7.72±0.18	7.88±0.10
CP	41.43±0.2	41.78±0.14	41.22±0.21	40.98±0.25
Ash	19.12±0.33	17.3±0.25	19.17±0.39	13.94±0.18
Crude Fat	4.98±0.34	5.16±0.32	4.2±0.15	4.9±0.21
Crude Fiber	7.13±0.12	6.97±0.14	7.01±0.31	7.21±0.42

1. CM fish meal® (CMFM) was procured from Cool man fishmeal factory, Thaleimannar road, Pesalei, Sri Lanka.
2. Chitosan extracted in laboratory of Institute of Post Harvest Technology in National Aquatic Resources Research and Development Agency (NARA), Sri Lanka.
3. 500g of Commercial chitosan also was imported from India (250 US$ per kg).
4. Supphaiah feed suppliers, Wolfendhal Street, Colombo 13, Sri Lanka
5. Local retail market
6. Contains (as mg/ kgin diets): Vitamin A, 9000IU; Vit. B_1, 2mg; Vit. B_2, 3.6mg; Vit. B_6, 1mg; Vit.B_{12}, 10mg; Vit. D_3, 2000IU; Vit. E, 5mg; Vit. K, 2mg; calcium pantothenate, 4mg; Choline chloride, 150mg; Folic acid, 0.5mg; Niacinamide, 16mg; Fe, 25mg; Mn, 60mg; Cu, 5mg; Zn, 50mg; I, 5.5mg; CO, 0.1mg.

Table 2: Growth performances of juvenile tiger shrimps fed four different diets (Means ± SE) [1].

	T_1	**T_2**	**T_3**	**T_4**
Initial weight	0.1	0.1	0.1	0.1
Final weight	0.171^{c}	0.205^{ab}	0.226^{a}	0.200^{b}
Percent weight gain [2]	70.90 ± 0.31^{c}	104.58 ± 3.76^{ab}	126.03 ± 10.81^{a}	99.74 ± 9.91^{b}
SGR [3]	1.28 ± 0.01^{c}	1.70 ± 0.04^{ab}	1.94 ± 0.12^{a}	1.64 ± 0.12^{b}
SR in the feeding trial	47.78±11.28	49.44±7.47	45±4.19	52.22±10.6
SR (%) during challenging [4]	38.29±9.7	49.97±11.68	64.14±17.38	34.05±7.94

1. Values are means from triplicate groups of fish, where the values in each row with different superscripts are significantly different ($P<0.05$).
2. Percent Weight gain = (final weight - initial weight) × 100 / initial weight.
3. SGR: specific growth rate (% day^{-1}) = ($\log_e$ final wt.-$\log_e$ initial wt.)/days*100
4. SR: survival rate (%) = (# of sea cucumber at the end of challenging /# of sea cucumber at the start of challenging) 100

5. Conclusion

Chitosan would be a good immunostimulant/ growth promoter in juvenile tiger shrimp diets. Even though, commercial chitosan which used in diet 3 and 4 is very expensive for using into shrimp diets. Therefore, the chitosan, which used in diet 2 can be extracted easily as a supplement to promote growth and boost the immune system in shrimp.

6. Acknowledgements

Authors thank Alpex marine company limited for supplying shrimp head waste to extract chitosan and Mr. Priyantha low, Ambakandawila, Chilaw, Sri Lanka for providing shrimp juvenile and the place to conduct the feeding trial.

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