



# Dose/frequency: A critical factor in the administration of glucan as immunostimulant to Indian white shrimp *Fenneropenaeus indicus*

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## ARTICLE INFO

### Article history:

Received 14 March 2008

Received in revised form 23 October 2008

Accepted 24 October 2008

### Keywords:

*Fenneropenaeus indicus*

*Candida sake*

Glucan

Immunostimulant

Dose

Frequency

Survival

## ABSTRACT

The immunostimulatory effect of an alkali insoluble glucan extracted from marine yeast isolate *Candida sake* S165 was tested in *Fenneropenaeus indicus*. Post larvae (PL) of *F. indicus*, fed glucan incorporated diet at varying concentrations (0.05, 0.1, 0.2, 0.3, 0.4 g glucan/100 g feed) for 21 days were challenged orally with white spot syndrome virus (WSSV). Maximum survival was observed in PL fed the 0.2% glucan incorporated diet. Subsequently the feed incorporated with 0.2% glucan was fed to *F. indicus* post larvae at different feeding intervals, i.e. daily, once every two days, once every five days, once every seven days and once every ten days. After 40 days, the prawns were challenged orally with WSSV and post challenge survival was recorded. Shrimp feed containing 0.2% glucan when administered once every seven days gave maximum survival. This was supported by haematological data obtained from adult *F. indicus*, i.e. total haemocyte count, phenoloxidase activity and nitroblue tetrazolium reduction (NBT). The present observation confirms the importance of dose and frequency of administration of immunostimulants in shrimp health management.

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## 1. Introduction

Outbreaks of diseases are being increasingly recognized as a significant constraint in aquaculture production. Among shrimp pathogens, white spot syndrome virus (WSSV) causes high mortality in cultured shrimp species viz. *Penaeus monodon*, *Fenneropenaeus indicus*, *Marsupenaeus japonicus* and *Penaeus semisulcatus* (Lightner, 1996; Lo et al., 1997). Polysaccharides from a variety of sources enhance the immune system of animals and pharmacologically they are known as biological response modifiers (BRMs) (Leung et al., 2006). The most active of these compounds is (1–3)- $\beta$ -D-glucan (Bhon and Be Miller, 1995), a natural polymer isolated from the cell wall of yeast and mold.  $\beta$ -glucans have been used as immunostimulants to enhance the defence potential of fish and shellfish against bacterial or viral infection (Oliver et al., 1986; Sung et al., 1994; Song et al., 1997; Chang et al., 1999, 2000, 2003). Currently many commercial immunostimulants are available in the shrimp aquaculture industry and are extensively used by shrimp farmers. However, scientific data in support of their function and dose/frequency of application are lacking. Information regarding the dose is essential as overdose leads to immunosuppression rendering less protection and animals succumb to infection. In the present study, the immunostimulatory effect of an alkali insoluble cell wall glucan preparation from a marine

yeast isolate *Candida sake* S165 (Sajeevan et al., 2006) was tested in *F. indicus* and the dose/frequency was optimised.

## 2. Materials and methods

### 2.1. Glucan extraction

*C. sake* S165, isolated from the coastal waters off Cochin and maintained in the Microbiology Laboratory of the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences was used for the study. A pure lawn culture of *C. sake* was prepared using Malt Extract Agar (malt extract, 20 g; mycological peptone, 5 g; agar, 20 g; 20‰ seawater 1 L, pH 6) and the biomass was harvested at exponential phase into sterile seawater (20‰). The harvested cells were then separated by centrifugation at 7500  $\times$ g for 10 min at 4 °C and dried at 80 °C for 24 h. Glucan was extracted from the dried yeast biomass following the method of Williams et al. (1991) with modification. Briefly, 1 g dried yeast biomass suspended in 20 mL 3% NaOH was maintained at 100 °C for 6 h in a serological water bath. Filtering through muslin silk and re-extracting with NaOH resulted in separation of an alkali insoluble material. The insoluble material was separated and extracted with 20 mL 0.5 N acetic acid at 75 °C for 6 h. The insoluble material was recovered by filtration through muslin silk and refluxed repeatedly with ethanol until the filtrate became colourless. The remaining precipitate was washed extensively with distilled water, vacuum dried over silica gel at 28  $\pm$  1 °C and the final product (glucan) was used for the study.

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## 2.2. Test for immunostimulatory potential of yeast cell wall glucan through oral administration

### 2.2.1. Experiment I: optimisation of dose

Glucan prepared from *C. sake* S165 was homogenised and coated on to a standard shrimp diet using a commercial binder “BINDEX” (Matrix Biosciences Ltd, Hyderabad). Five test feeds were prepared with varying glucan concentrations, i.e. 0.05, 0.1, 0.2, 0.3 and 0.4 g glucan/100 g feed. Feed without glucan was used as the control. All preparations were stored at  $-20^{\circ}\text{C}$  until used.

**2.2.1.1. Experimental design.** *F. indicus* post larvae, PL20 (PCR-negative for WSSV) brought from a shrimp hatchery located at Kannamali, Cochin and acclimatized to laboratory conditions for one week were used for the study. Twenty five *F. indicus* post larvae were stocked in fibre reinforced plastic tanks containing well aerated 15‰ seawater. Feeding experiments were done in triplicate with experimental feeds along with the control diet for a period of 21 days. Feeding was performed twice daily (0800 and 1900 h) at a ration of 10–15% body weight. Water quality parameters of the rearing water (salinity,  $\text{NH}_3\text{-N}$ ,  $\text{NO}_2\text{-N}$ ,  $\text{NO}_3\text{-N}$  and dissolved oxygen) were monitored regularly (APHA, 1995) and maintained at optimal levels by water exchange. The post larvae were challenged with white spot syndrome virus (WSSV) by feeding WSSV infected shrimp (*F. indicus*) tissue (1 g/25 post larvae) on the 22nd day. The percentage survival of the post larvae in each of the experimental groups was recorded up to the 7th day of challenge.

### 2.2.2. Experiment II: optimisation of frequency of administration of glucan through feed

Based on experiment I, the 0.2% glucan diet was selected for further study. Post larvae (PL 25) of *F. indicus*, (PCR-negative for WSSV) were used for the study. Rearing conditions of larvae were maintained as in experiment I. The post larvae were divided into six groups and each group was fed the 0.2% glucan diet at different frequencies viz., daily (G1), once every two days (G2), once every five days (G5), once every seven days (G7) and once every ten days (G10). The control diet was fed on the rest of the days to all the treatment groups. After 40 days, all treatment groups were challenged with WSSV via diet as described in Section 2.2.1. The percentage survival of the animals in each of the experimental groups was recorded up to the 7th day of challenge.

### 2.2.3. Experiment III: confirmation of optimal dose and frequency through immune assay

Apparently healthy adult *F. indicus* (mean body weight  $23 \pm 1$  g) were used for the study. The shrimp, after seven days quarantine, were randomly divided into four groups of 60 shrimp per aquarium of 500 L capacity and acclimatized to the laboratory conditions for one week.

Among the four groups, the first group was fed the basal diet devoid of glucan and treated as the control. The other groups were maintained on the 0.2% glucan diets at definite feeding intervals, i.e. daily (G1); once every seven days (G7) and once every ten days (G10). All test groups were fed the control diet for the remaining days. Rearing conditions and water quality parameters were maintained as per Section 2.2.1.1. On the 41st day, the shrimps were challenged with white spot syndrome virus by feeding WSSV infected prawn tissue at the rate of 1 g/shrimp. Afterwards they were maintained on their respective experimental diets and the immune parameters were assayed at definite intervals. The post challenge survival percentage in each experimental group was also recorded for a period of seven days.

## 2.3. Assay of immunological parameters

Haemolymph was withdrawn aseptically from the rostral sinus using specially designed sterile capillary tubes having a diameter of 0.5 mm, pre-rinsed with anticoagulant (0.01 M Tris HCl, 0.25 M sucrose, 0.1 M trisodium citrate, prepared in double distilled

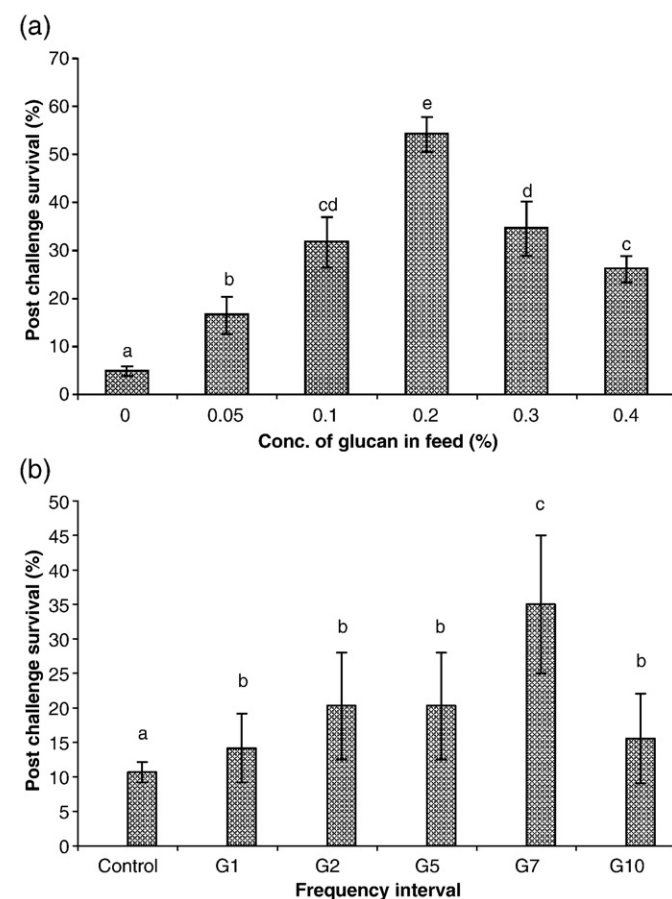
water, autoclaved and adjusted to pH 7.6; Song and Hsieh, 1994) and transferred to a sterile microcentrifuge tube containing cooled anticoagulant. The haemolymph collected from four shrimp ( $n=4$ ) of each group was assayed separately. Sampling was done at the beginning of the feeding experiment, day 0 (base line), day 40, and post-challenge day 1 (PCD1), 2 (PCD2) and 3 (PCD3). Total haemocyte count (THC) was made using a Neubauer improved haemocytometer and expressed as  $\text{THC mL}^{-1}$  haemolymph. Phenoloxidase (PO) activity was measured spectrophotometrically by using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate (Soderhall, 1981). The dopa-chrome formed was measured at 495 nm and phenoloxidase activity was then expressed as the increase in absorbance per min per 100  $\mu\text{L}$  haemolymph. Respiratory burst activity of haemocytes was measured spectrophotometrically (Song and Hsieh, 1994) using nitroblue tetrazolium (NBT) as the substrate. The blue formazan formed due to  $\text{O}_2^-$  reduction during phagocytosis of haemocytes was measured at 620 nm and expressed as NBT activity per 100  $\mu\text{L}$  haemolymph.

## 2.4. Statistical analysis

In order to determine significant difference, the results were analyzed using one way analysis of variance (ANOVA) and Duncan's multiple comparison of the means using SPSS 10.0 for Windows. Differences were considered significant at  $P<0.05$ .

## 3. Results and discussion

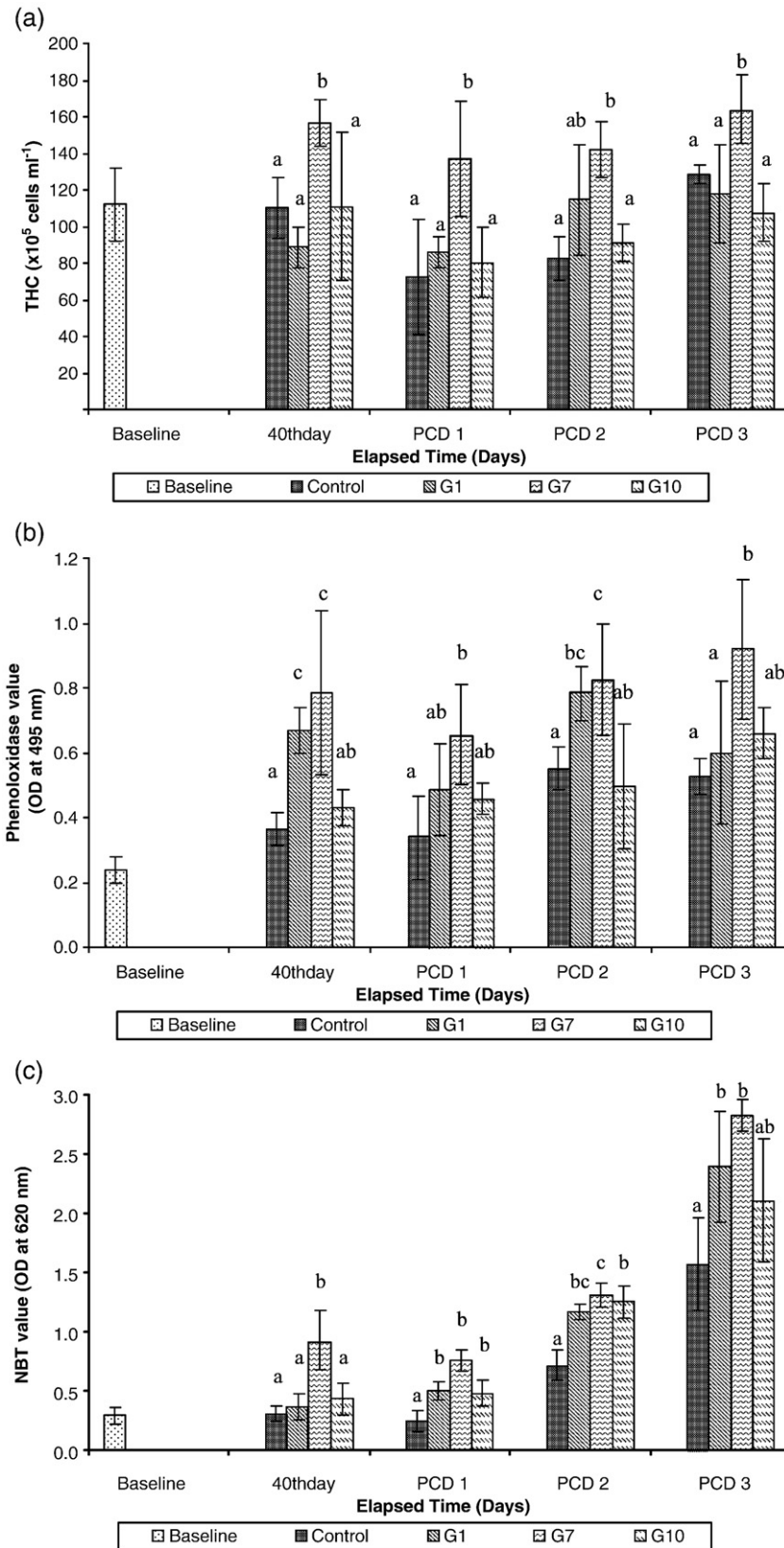
The alkali extraction of particulate glucan from the cell wall of the marine yeast *C. sake* S165 was found to give a yield of 12.3% of the dry



**Fig. 1.** Post challenge survival (on the 7th day) in *F. indicus* post larvae challenged with WSSV after feeding *C. sake* glucan-incorporated diet (a) different concentrations (b) at different frequency intervals. Values with the same superscript do not differ significantly ( $P<0.05$ ).

weight of the yeast biomass. After feeding the glucan containing diets, a glucan content of 0.2% was judged optimal based on the highest protection against WSSV during experimental infection. Post

challenge survival against WSSV on the 7th day with the 0.2% glucan ( $54.17 \pm 3.62\%$ ) feed was found to be significantly different from the other treated groups including the control ( $4.74 \pm 0.99\%$ ). Both the



**Fig. 2.** The immunological profile of *F. indicus* fed diets containing glucan at different frequencies for 40 days and then challenged with WSSV. (a) Total haemocytosis, (b) phenoloxidase (PO) value and (c) NBT value. Data at the same exposure time with different letters are significantly different ( $P < 0.05$ ). G1 = glucan feed daily, G7 = glucan feed once every seven days, and G10 = glucan feed once every ten days. PCD = Post-challenge day.

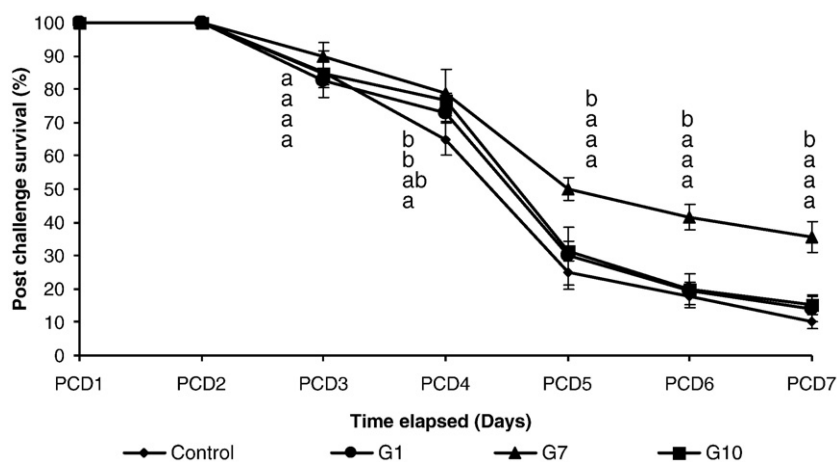


Fig. 3. Mean ( $\pm$ S.D.) post challenge survival of *F. indicus* fed glucan diets at different frequencies for 40 days against experimental infection of WSSV. Data at the same exposure time with different letters are significantly different ( $P < 0.05$ ).

higher (0.3 and 0.4%) and lower (0.05 and 0.1%) glucan diets offered less protection to the animals (Fig. 1a), but significantly higher than the control group.

Post larvae, which were fed the glucan diet once every seven days (G7) showed higher ( $P < 0.05$ ) survival ( $35 \pm 10\%$ ) against WSSV challenge compared to the other groups and the control ( $10.67 \pm 1.47\%$ ) (Fig. 1b). The immunological profile of the prawns in all treatment groups showed an increasing trend just after challenge. The highest THC, PO and NBT values were observed on PCD3 (Fig. 2a–c). The shrimps fed 0.2% glucan at a feeding frequency of once every seven days showed significantly higher immune response compared to the control and other treatment groups ( $P < 0.05$ ). This was supported by the better post challenge survival observed in this group (Fig. 3). These observations emphasize the importance of dose/frequency of application of immunostimulants in shrimp farming for imparting protection against pathogens.

The reduced immunity index in daily glucan fed shrimp (G1) could be due to an overdose of glucan by this group. Continuous intake of the glucan diet for a period of 40 days might have caused an immune fatigue in this group. Shrimp fed the glucan diet once every ten days (G10) showed a low immune profile and survival upon WSSV challenge. This might be due to a suboptimal level of glucan received by this group.

Unlike many chemotherapeutics, immunostimulants do not show a linear dose–effect relationship (Bliznakov and Adler, 1972). In fact they often show a distinct maximum at a certain intermediate concentration and even a complete absence of effect or an adverse toxic effect at higher concentrations (Floch et al., 1987). It presumes that higher concentrations of glucan caused excessive degranulation of both granular and semigranular haemocytes resulting in the release of prophenoloxidase and an exhaustion of immune system. A higher dose of glucan in feed for longer period may result in immunosuppression and it is more likely that such animals may not survive infection by potent pathogens and succumb to death following an infection. Host homeostasis seems to be impaired due to high concentrations of glucan, a condition very similar to an acute infection by a pathogen leading to the reduction in circulating haemocytes. In such situations the mechanism to restore the haemocyte number is mobilizing the haemocytes from the reservoirs in haemal crypts or upregulation of cell division in the haematopoietic tissue and proliferation within the haemolymph (Chiretti-Magalda et al., 1977; Hose et al., 1992). Soderhall et al. (2003) reported that injection of  $\beta$ 1,3-glucan into the haemocoel of a crustacean resulted in an accelerated maturation of haemocyte precursors in the haematopoie-

tic tissue followed by release of new cells into the circulation. However, it is not clear to what extent the new cells produced in this way are immunologically mature or competent and how long it will take for the complete restoration of immune equilibrium (Smith et al., 2003). Chang et al. (2000) reported a reduced immunity in *P. monodon* after continuous feeding of glucan at a concentration of  $2 \text{ g kg}^{-1}$  feed for 40 days. These results suggest that continuous use of immunostimulants even at an optimal dose may suppress the immunity in shrimp and administration at definite intervals would give better performance resulting in enhanced survival. The low survival of shrimp at higher concentrations of glucan also could be attributed to the continuous overproduction of superoxide anions and free radicals generated from the respiratory burst activity of the phagocytic haemocytes, causing non-specific host injury. Our earlier study with a marine yeast diet as an immunostimulant in *F. indicus* showed that a 10% yeast diet was optimal whereas, higher doses resulted in a lower immune profile and post challenge survival (Sajeevan et al., 2006).

The short lived nature of protection, lack of linear dose–effect relationship and lack of immune memory make the regimes of immunostimulant application in crustaceans complicated. The present observations highlight the importance of the rational use of immunostimulants in shrimp culture. Indiscriminate use of an immunostimulant without knowing the optimum dose and frequency could never be an effective management strategy. Furthermore, such prophylactic measures may result in impaired immunity in animals and in the event of an infection, they may easily succumb to death. This study emphasizes the fact that the dose and frequency of application of immunostimulants in shrimp aquaculture should be standardised and validated before commercialisation to achieve optimum stimulation of the immune system and to avoid immune fatigue due to overdose. In the present study, even though the glucan incorporated diet did not provide total protection for *F. indicus* against WSSV, the partial protection would help the aquaculture industry to plan for a prior harvest and to save the crop from mass mortality.

#### Acknowledgement

The authors are grateful to the Department of Ocean Development (DOD), Govt. of India for a research grant (F.No.DOD/II-MRDF/4/3/UNI/97-(P-6)) with which the work was carried out. We thank R. Bernard (University of Kentucky) and R. Rajesh (University of Michigan) for critical evaluation of the manuscript. The first author gratefully acknowledges DOD for the fellowship rendered.

## References

- APHA, 1995. In: Eaton, A.D., Clescenri, L.S., Greenberg, A.E. (Eds.), Standard Methods for the Examination of Water and Wastewater, 19th ed. American Public Health Association, Washington DC, USA.
- Bhon, J.A., Be Miller, J.N., 1995. (1–3)- $\beta$ -D-glucan as biological response modifiers: a review of structure–function activity relationships. *Carbohydr. Polym.* 28, 3–14.
- Bliznakov, E.G., Adler, A.D., 1972. Non-linear response of the reticuloendothelial system upon stimulation. *Pathol. Microbiol.* 38, 393–410.
- Chang, C.F., Su, M.S., Chen, H.Y., Lo, C.F., Kou, G.H., Liao, I.C., 1999. Effect of dietary beta-1,3-glucan on resistance to white spot syndrome virus (WSSV) in post larval and juvenile *Penaeus monodon*. *Dis. Aquat. Org.* 36, 163–168.
- Chang, C.F., Chen, H.Y., Su, M.S., Liao, I.C., 2000. Immunomodulation by dietary  $\beta$ -1,3-glucan in the brooders of the black tiger shrimp, *Penaeus monodon*. *Fish Shellfish Immunol.* 10, 505–514.
- Chang, C.F., Su, M.S., Chen, H.Y., Liao, I.C., 2003. Dietary  $\beta$ -1,3-glucan effectively improve immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. *Fish Shellfish Immunol.* 15, 297–310.
- Floch, F., Poirier, J., Fizames, C., Pimelautide, R.W., 1987. From experimental result to clinical trials: an illustration. In: Azuma, I., Jolles, G., (Eds.) *Immunostimulants: Now and Tomorrow*. Tokyo/Springer-Verlag, Berlin: Japan Science Society Press, pp. 183–204.
- Ghiretti-Magaldi, A., Milanesi, C., Tognon, G., 1977. Haemopoiesis in Crustacea Decapoda. Origin and evolution of haemocytes and cyanocytes of *Carcinus maenas*. *Cell Differ. Dev.* 6, 167–186.
- Hose, J.E., Martin, G.G., Tiu, S., Mckrell, N., 1992. Pattern of haemocytes production and release throughout the moult cycle in the penaeid shrimp, *Sicyonia ingentis*. *Biol. Bull.* 183, 185–199.
- Leung, M., Liu, C., Koon, J., Fung, K., 2006. Polysaccharide biological response modifiers. *Immunol. Lett.* 105, 101–114.
- Lightner, D.V., 1996. A Handbook of Pathology and Diagnosis Procedures for Disease of Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA.
- Lo, C.F., Lin, K.F., Ho, C.H., Chu, Y.L., Chen, C.H., Yeh, P.Y., Peng, S.E., 1997. Detection and tissue tropism of white spot syndrome baculo virus (WSBV) in captured brooders of *P. monodon* with a special emphasis on reproductive organs. *Dis. Aquat. Org.* 30, 53–72.
- Oliver, G., Eaton, C.A., Campell, N., 1986. Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). *Vet. Immunol. Immunopathol.* 12, 223–234.
- Sajeevan, T.P., Rosamma Philip, I.S., Bright, Singh, 2006. Immunostimulatory effect of a marine yeast *Candida sake* S165 in *Fenneropenaeus indicus*. *Aquaculture* 257, 150–155.
- Smith, V.J., Brown, J.H., Hauton, C., 2003. Immunostimulation in crustacean: does it really protect against infection? *Fish Shellfish Immunol.* 15, 71–90.
- Soderhall, K., 1981. Fungal cell wall  $\beta$ -1–3 glucans induce clotting and phenoloxidase attachment to foreign surfaces of crayfish haemocyte lysate. *Dev. Comp. Immunol.* 5, 565–573.
- Soderhall, I., Bangyeekhun, E., Mayo, S., Soderhall, K., 2003. Haemocyte production and maturation in an invertebrate animal: proliferation and gene expression in haematopoietic stem cell of *Pacifastacus leniusculus*. *Dev. Comp. Immunol.* 27, 661–672.
- Song, Y.L., Hsieh, Y.T., 1994. Immunostimulation of tiger shrimp (*Penaeus monodon*) haemocytes for generation of microbicidal substances: analysis of reactive oxygen species. *Dev. Comp. Immunol.* 18, 201–209.
- Song, Y.L., Liu, J.J., Chan, L.C., Sung, H.H., 1997. Glucan induced disease resistance in tiger shrimp (*Penaeus monodon*). *Fish Vaccinology. Dev. Biol. Stand.* 90, 413–421.
- Sung, H.H., Kou, G.H., Song, Y.L., 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). *Fish Pathol.* 29, 11–17.
- Williams, D.L., McNamee, R.B., Jones, E.L., Pretus, H.A., Ensley, H.E., Browder, I.W., Di Luzio, N.R., 1991. A method for the solubilisation of a (1–3)- $\beta$ -D-glucan isolated from *Saccharomyces cerevisiae*. *Carbohydr. Res.* 219, 203–213.