EXPRESSION OF IMMUNO-RELATED GENE (LYSOZYME) AFTER USING IMMUNOSTIMULANT IN KURUMA SHRIMP (*Marsupenaeus japonicus*)

Vu Duc Hanh[1*], Nguyen Thi Hong Chien[1], Dao Le Anh[1], Nguyen Thi Hang[1], Le Van Truong[1], T. Itami[2], Lai Thi Lan Huong[1], Nguyen Thi Lan[1]

[1]Faculty of Veterinary Medicine, Vietnam National University of Agriculture
[2]Faculty of Agriculture, University of Miyazaki, Japan

Email[*]: Vuduchanh216@gmail.com

Received date: 23.02.2017 **Accepted date: 01.04.2017**

ABSTRACT

Immunostimulants are the substances, which enhance the immuno-defense activity and provide resistance to fish and shrimp diseases. In order to clarify the lysozyme expression of kuruma shrimp (*Marsupenaeus japonicus*) after feeding immunostimulant, the supplement containing microalgae and Euglena (Ex400) were selected for the trials. Total RNA was extracted by RNA Iso Plus from the lymphoid organ, intestine and blood of shrimp that was fed with Ex400 diet. Tissues were sampled at 0, 3, 7, and 10 days after feeding. Lysozyme transcripts significantly increased in Ex400-fed shrimps compared to control diet-fed shrimps ($P < 0.01$) at 7 and 10 days post-feeding in lymphoid organ and intestine. Lysozyme expression of blood of shrimp also exhibited higher level than those of control at 3 and 10 days post feeding ($P < 0.01$). In conclusion, the result of lysozyme gene expression suggested that immunostimulant could activate lysozyme in the immune system of kuruma shimp.

Keywords: Gene expression, immunostimulants, kuruma shrimp, lysozyme, quantitative real-time PCR.

Biểu hiện của lysozyme gen liên quan đến miễn dịch sau khi sử dụng chất kích thích miễn dịch trong tôm he Nhật Bản

TÓM TẮT

Chất kích thích miễn dịch là chất giúp tăng cường hoạt động của hàng rào miễn dịch và cung cấp sức đề kháng với bệnh. Ex400 được sản xuất từ vi tảo và Euglena là chất kích thích miễn dịch trên tôm được sử dụng để xác định biểu hiện gen của lysozyme trong tôm he Nhật Bản. ARN tổng số được chiết xuất từ các cơ quan lympho, ruột và máu của tôm tại các thời điểm 0, 3, 7 và 10 ngày sau khi cho tôm ăn Ex400. Trong ruột và cơ quan lympho của tôm, biểu hiện gen của lysozyme cao hơn so với lô đối chứng tại các thời điểm 7 và 10 ngày sau khi cho tôm ăn Ex400 ($P< 0,01$). Biểu hiện lysozyme trong máu của tôm cũng được xác định ở mức độ cao hơn so với lô đối chứng tại thời điểm 3 và 10 ngày sau khi cho ăn Ex400 ($P < 0,01$). Kết quả mức độ biểu hiện gen của lysozyme cho thấy chất kích thích miễn dịch có thể kích hoạt lysozyme trong hệ thống miễn dịch của tôm he Nhật Bản..

Từ khóa: Biểu hiện gen, chất kích thích miễn dịch, định lượng real-time PCR, tôm Kurumalysozyme.

1. INTRODUCTION

Shrimp culture is an important source of livelihood of people in some countries. The production of cultivated penaeid shrimp species increased exponentially since the early 1970s. However, there is a rapiddly increasing problem with serious disease outbreaks. As shrimps lack an adaptive immune system, they rely on innate immune responses against microbial invasion (Tanekhy and Fall, 2015). A better understanding of the innate immune system of shrimp will undoubtedly help us to develop strategies in disease control and sustainable shrimp farming.

Lysozyme is one of the earliest known antibacterial proteins, omnipresent among eukaryotes and prokaryotes (Tyagi *et al.*, 2007). It is an important component of the non-specific innate immune system to provide protection from microbial infections by degrading the β-1,4 glycosidic bond between *N-acetylmuramic acid* and *N acetylglucosamine* in the peptidoglycan of bacterial cell walls (Ringø, 1993).

Immunostimulants are substances that activate the immune system of animals to make them more resistant to microbial infections (Raa, 1996). The definition has been expanded somewhat to include live organisms or their products that have an impact on the immune system. The use of immunostimulants does not generate a specific response to a certain antigen, but causes an overall response that hastens recognition and elimination of a broad range of infectious agents and foreign substances (Sordillo *et al.*, 1997). The present study was carried out to examine the expression of this lysozyme after using immunostimulant containing microalgae and Euglena (Ex400) in Kuruma shrimp (*Marsupenaeus japonicus).*

2. MATERIALS AND METHODS

2.1. Animals

Specific pathogen free (SPF) kuruma shrimps, *Marsupenaeus japonicus,* of 10 ± 0.3 g body weight were obtained from Matsumoto Fisheries, Miyazaki, Japan.

2.2. Methods

2.2.1. Experimental design

Prior to feeding experiment, shrimp were acclimatized, reared in aerated seawater tank at 23°C and 30 ppt salinity, and fed with control diet in 3 days. After that, each group of 12 shrimps was fed with immunostimulant containing microalgae and euglena (Ex400). At each indicated condition, the control group was fed with control diet. The shrimps were fed with Ex400 and control diet at 1.5% body weight per day for 10 days. Three shrimp were collected at 0, 3, 7, and 10 day for experiment. The experiment was conducted in triplicate. Shrimp body surfaces were washed and disinfected with 70% ethanol, and then the blood, intestine, lymphoid organ were dissected out. One side of lymphoid organ, 200µL of blood, and 1/10 of gut were collected.

2.2.2. RNA extraction

Total RNA was extracted from the sample using RNA Iso plus (TAKARA, Japan). According to the manufacturers' instruction, the quantity and quality of all RNA samples were checked using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE, USA) at 260nm and 280nm..

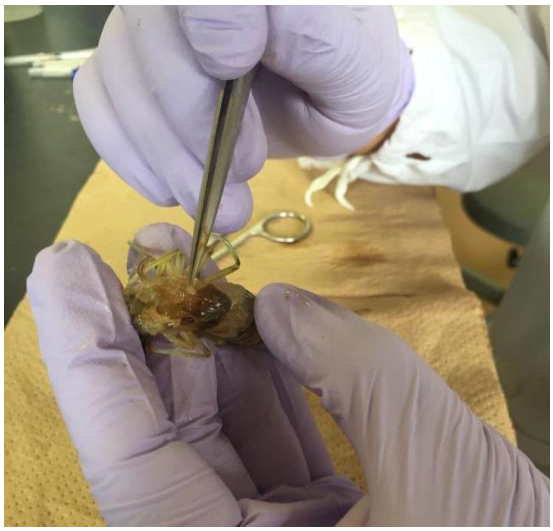
2.2.3. Synthesis of cDNA

cDNA was synthesized according to the protocol (TOYOBO, Japan) of ReverTra Ace qPCR RT Master Mix with gDNA Remover, using RNA solution resulted from RNA extraction protocol. Nuclease-free water was added to RNA template. Thermal cycler condition was 65°C for 5 minutes and after rapid cooling on ice 2µl of 4×DN Master Mix (gDNA Remover) were added. The thermal cycler profile was 37°C for 10 minutes, then 2µl of 5×RT Master Mix were added to the mix. The thermal cycler condition for the 3rd step of PCR (reverse transcription reaction) was 37°C for 15 minutes 98°C for 5 minutes. cDNA was used as a template for real-time PCR analysis.

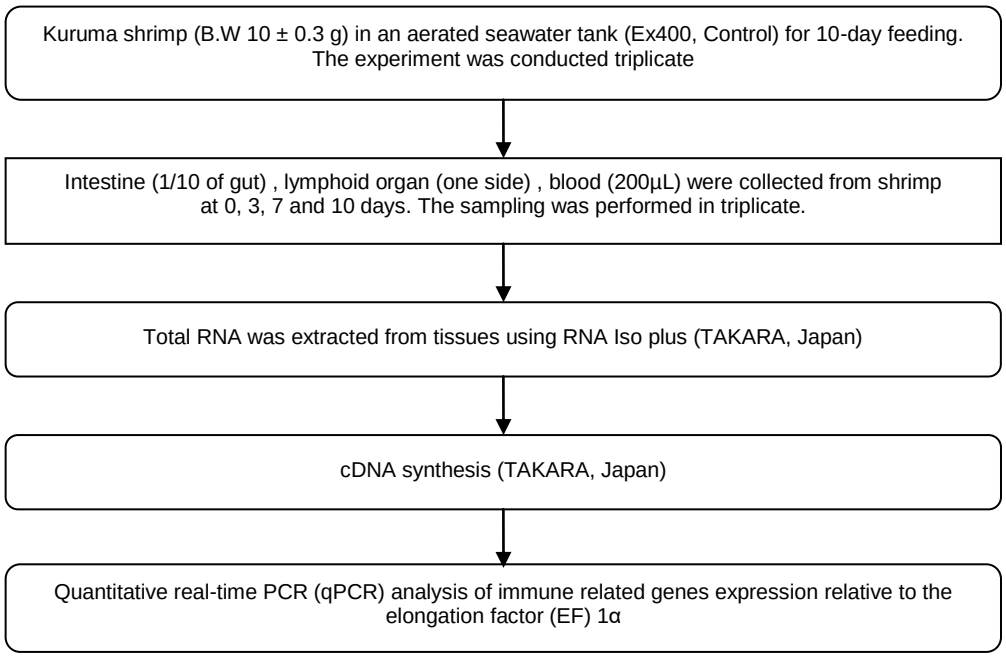
2.2.4. Quantitative RT-PCR for determining gene expression

A qRT-PCR on cDNA specimens was performed using SYBR Green Master Mix (Applied Biosystems). Elongation factor EF-1α gene was used as an internal control. The EF-1α and their respective primers are presented in table 1. All PCR reactions were performed in a reaction mixture containing 10.4µL of SYBR Green Master Mix, 4µL of 10pM primer set (lysozyme/EF-1α), 2µL of template DNA(10ng), and 3.6µL of nuclease-free water.

Picture 1. Blood collection



Picture 2. Lymphoid organ collection



Picture 3. Diagram of experimental design

Table 1. The primers used to amplify EF-1 α and Lysozyme of kuruma shrimp

Gene	Sequence (5' to 3')	Accession number
[a]EF-1 α- Forward	TTCGCTGAACTGCTGACCAA	AB458256
[a]EF-1 α- Reverse	GCTTGCTGGGAACCATCTTG	
Lysozyme- Forward	GCGTGGACTACGGCATCTTC	AB080238
Lysozyme- Reverse	GAGATCGGAGCATGGGATTC	

Note: [a] EF-1α specific primer were taken from a previous publication
Source: Maeda et al., 2014

Amplification was carried out as follows: 60s at 95°C, 40 cycles of 15s at 95°C, and 40s at 60°C. Thermal cycling and fluorescence detection were conducted using RT-PCR system (Applied Biosystem) with detection run in duplicate. The threshold cycle (C_T) representing the PCR cycle at which an increase in reporter fluorescence above signal was first detected. The comparative C_T method $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was used to analyze the expression level of the shrimp genes.

2.3. Statistical analysis

Analysis of variance was carried out using SPSS statistics version 18, to see the significance of expression of the gene at various time points. Independent t-test was performed to see significance in expression between Ex400 fed shrimp and control diet-fed shrimp.

3. RESULTS AND DISCUSSION

3.1. Gene expression of lysozyme in blood

After 0, 3, 7 and 10 days of feeding experiment, 12 shrimps from each tank (Ex400 and Control) were collected. The level of lysozyme expression of control and Ex400 of the Ex supplementation is given in Fig. 1. In blood, the transcript level of lysozyme in Ex400-fed shrimps was significantly higher ($P < 0.01$) than control diet-fed shrimps at 3 and 10 days post feeding.

3.2. Lysozyme expression in intestine

After 0, 3, 7 and 10 days of feeding experiment, 12 shrimps from each tank (Ex400 and control) were collected. Lysozyme transcripts significantly increased in Ex400-fed shrimps compared to control diet-fed shrimps ($P < 0.05$ at 3 days post feeding) and ($P < 0.01$ at 7, 10 days post feeding).

3.3. Lysozyme expression gene in lymphoid organ

After 0, 3, 7 and 10 days of feeding experiment, 12 shrimps from each tank (Ex400 and Control) were collected. At 3 days post feeding, although higher expression of lysozyme was observed in lymphoid organ of Ex400-fed shrimps, the expression level was not different ($P < 0.05$) from those of the control diet-fed shrimps. Lysozyme transcripts significantly increased in Ex400-fed shrimps compared to control diet-fed shrimps ($P < 0.01$) at 7 and 10 days post-feeding.

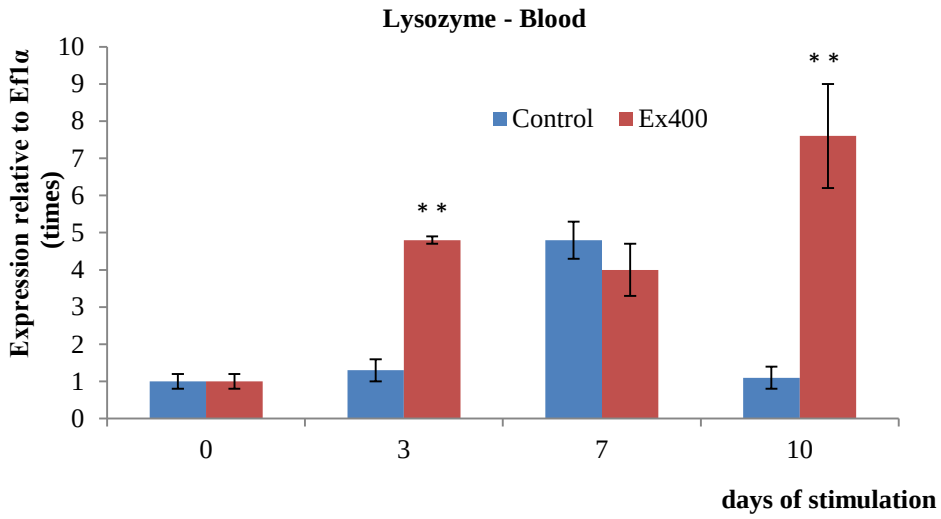


Fig.1. Quantitative real-time PCR analysis of lysozyme expression relative to (EF)-1α gene trancript in blood of control and Ex400 diet-fed kuruma shrimp

*Note: Data are presented as mean ± SD. Differences were considered significant at $P < 0.05$ and $P < 0.01$ as indicated by asterisk * and **, respectively*

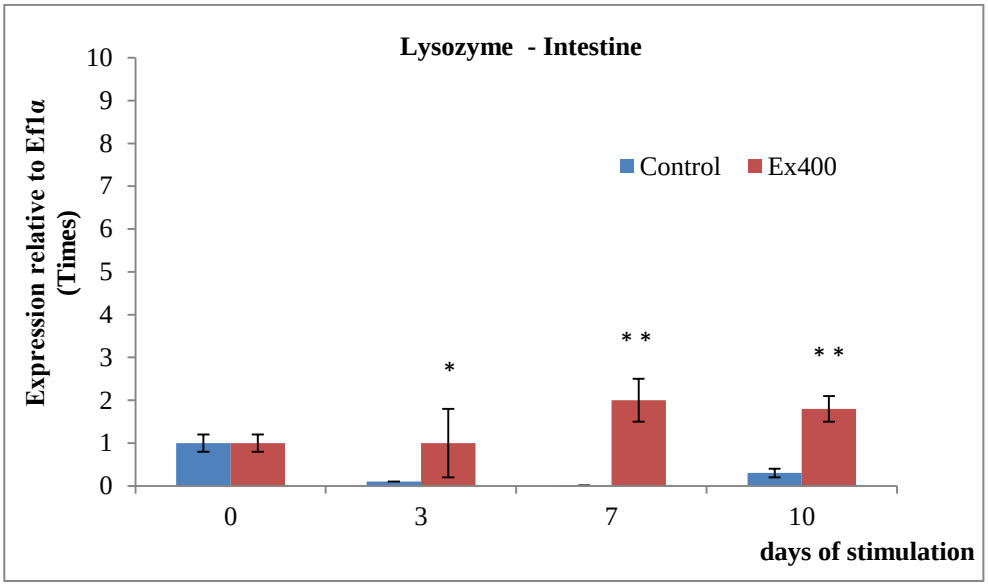


Fig. 2. Quantitative real-time PCR analysis of Lysozyme expression relative to (EF)-1α gene trancript in the intestine of control and Ex400 diet-fed kuruma shrimp

*Note: Data are presented as mean ± SD. Differences were considered significant at P < 0.05 and P < 0.01 as indicated by asterisk * and **, respectively*

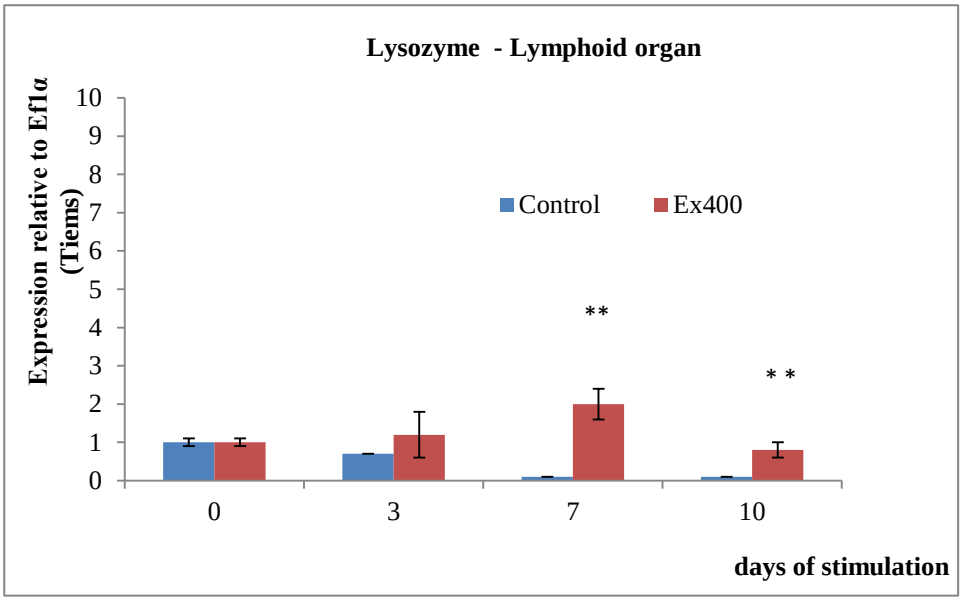


Fig 3 Quantitative real-time PCR analysis of Lysozyme gene expression relative to (EF)-1α gene trancript in the lymphoid organ of control and Ex400 diet-fed kuruma shrimp

*Note: Data are presented as mean ± SD. Differences were considered significant at P < 0.05 and P < 0.01 as indicated by asterisk * and **, respectively*

Lysozyme catalyzed the hydrolysis of bacterial cell wall and acts as a non specific innate immunity molecule upon invasion of bacterial pathogens (Jollès and Jollès, 1984). Lysozyme-like activity was determined in Chinese shrimp haemocytes 2 days after immune stimulation with laminarin (Mekata *et al.*, 2008). (Mekata *et al.*, 2008). Recently, it was

found that the expression of lysozyme significantly increased in the hemocyte of shrimps with immunostimulant (YK-6) in a 60 day experiment (Deng *et al.*, 2013). These results are consistent with our study in which expression analysis revealed that lysozyme was significantly increased after 3 and 10 days post feeding. At 7 days post feeding, although higher expression of lysozyme was observed in blood of Ex400-fed shrimps, the expression level was not different ($P > 0.05$) from those of the control diet-fed shrimps. All these results suggest that lysozyme is an important component of the shrimp defense system.

Moreover, an earlier *in situ* hybridization analysis showed that shrimp lysozyme was expressed mainly in granular and semigranular hemocytes and suggested that lysozyme was expressed in most if not all hemocytes in circulation and in peripheral tissues (intestine, lymphoid organ…) (Burge *et al.*, 2007). The intestine was a favorable site for invasion of pathogens carried in the water, food, and sediment (Jayabalan *et al.*, 1982). It was previously demonstrated that an influx of hemocytes entered the intestine of Penaeus monodon following exposure to Vibrio harveyi. Besides, the hemocytes associated with the basal lamina of S. ingentis were reported to fight pathogens entering the body via the midgut (Liuxy *et al.*, 1996). Therefore, intestine which have immune functions in immune system effectively protects against pathogens. The lymphoid organ, first described in *Penaeus orientalis* (Oka, 1969), which consisted of folded tubules with a central hemal lumen and a wall, layered with cells, was a site of bacterial uptake and phagocytosis by hemocytes (Van de Braak *et al.*, 2002). In most crustaceans, such as crabs and lobsters, that do not possess the lymphoid organ, phagocytes are involved in the uptake of foreign materials (Johnson, 1987). However, in those that do possess a lymphoid organ, including shrimps, it is the main site of bacteriostasis (Burgents *et al.*, 2005). The results showed that the expression of lysozyme was significantly increased in lymphoid organ and intestine of kuruma shrimp after DNA vaccination (Kono *et al.*, 2010). Recently, it has been demonstrated the lysozyme expression was significantly increased at 3 hpi only in both the lymphoid organ and intestine as compared to the control group (Tanekhy and Fall, 2015). In the present study, the expression level of lysozyme in the intestine and lymphoid organ were significantly increase at 3, 7 and 10 days after feeding as compared to control group.

In addition, the Ex400 diet test containing Euglena produced beta-glucan. Beta-glucan is able to activate phagocytes effectively in invertebrates. According to a previous study, shrimp fed with peptidoglycan-supplemented feed showed better growth and feed conversion rates than those fed a normal diet, and demonstrated that black tiger shrimp grew faster with glucan immersion which could be attributed to the higher activity of glucan delivered by immersion compared to oral administration (Boonyaratpalin *et al.*, 1995)

In this context, we observed the up-regulated lysozyme transcription in blood, intestine, lymphoid organ of Ex400-fed shrimps at 3, 7 and 10 days post-feeding, respectively. This result coupled with our findings, therefore, indicates that lysozyme is elicited by immunostimulating substances and acts as an integral component of the shrimp antibacterial defense mechanism.

On the basis of result, it will be of great interest to determine the gene expression, such as ALF, Crustins, Penaeidins, Toll receptor in kuruma shrimp in response to an *in vivo* stimulation and its resistance against viruses or bacteria.

4. CONCLUSIONS

Lysozyme transcripts were significantly increased by Ex400-fed shrimps compared to control diet-fed shrimps ($P < 0.01$) at 7 and 10 days post-feeding in lymphoid organ and intestine. Lysozyme transcripts in Ex400 fed shrimps was significantly higher ($P < 0.01$ at 3 and 10 days post-feeding) than control-diet-fed

shrimps in blood. The expression level at 10 days post-feeding was the highest in shrimp fed by Ex400 in blood. In conclusion, the result of lysozyme gene expression suggested that immunostimulant could activate lysozyme in the immune system of kuruma shimp.

REFERENCES

Boonyaratpalin, S., Boonyaratpalin, M., Supamattaya, K., and Toride, Y. (1995). Effects of peptidoglycan (PG) on growth, survival, immune response, and tolerance to stress in black tiger shrimp, Penaeus monodon. Diseases in Asian Aquaculture II. Fish Health Section. Manila, Philippines: Asian Fisheries Society, 469 - 477.

Burge, E. J., Madigan, D. J., Burnett, L. E., & Burnett, K. G. (2007). Lysozyme gene expression by hemocytes of Pacific white shrimp, Litopenaeus vannamei, after injection with Vibrio. Fish & shellfish immunology, 22(4): 327-339.

Burgents, J. E., Burnett, L. E., Stabb, E. V., and Burnett, K. G. (2005). Localization and bacteriostasis of Vibrio introduced into the Pacific white shrimp, Litopenaeus vannamei. Developmental and Comparative Immunology, 29(8): 681 - 691.

Cerezuela, R., Guardiola, F. A., Meseguer, J., and Esteban, M. A. (2012). Enrichment of gilthead seabream (*Sparus aurata* L.) diet with microalgae: effects on the immune system. Fish physiology and biochemistry, 38(6): 1729-1739.

Deng, D., Mei, C., Mai, K., Tan, B. P., Ai, Q., and Ma, H. (2013). Effects of a yeast-based additive on growth and immune responses of white shrimp, Litopenaeus vannamei (Boone, 1931), and aquaculture environment. Aquaculture Research, 44(9): 1348 - 1357.

Jayabalan, N., Chandran, R., Sivakumar, V., and Ramamoorthi, K. (1982). Occurrence of luminescent bacteria in sediments: current science assn cv raman avenue, po box 8005, bangalore 560 080, India, 51: 710 - 711.

Johnson, P. T. (1987). A review of fixed phagocytic and pinocytotic cells of decapod crustaceans, with remarks on hemocytes. Developmental and Comparative Immunology, 11(4): 679 - 704.

Jollès, P., and Jollès, J. (1984). What's new in lysozyme research? Molecular and cellular biochemistry, 63(2): 165 - 189.

Kono, T., Fall, J., Korenaga, H., Takayama, H., Iizasa, T., Mekata, T., . . . Sakai, M. (2010). Immunomodulation by DNA vaccination against white spot syndrome virus (WSSV). Asian Fisheries Science, 23(4): 435 - 446.

Liuxy, P. C., Lee, K. K., & Chen, S. N. (1996). Pathogenicity of different isolates of Vibrio harveyi in tiger prawn, Penaeus monodon. Letters in Applied Microbiology, 22(6): 413 - 416.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. methods, 25(4): 402 - 408.

Maeda, M., Shibata, A., Biswas, G., Korenaga, H., Kono, T., Itami, T., & Sakai, M. (2014). Isolation of lactic acid bacteria from kuruma shrimp (Marsupenaeus japonicus) intestine and assessment of immunomodulatory role of a selected strain as probiotic. Marine biotechnology, 16(2): 181 - 192.

Mekata, T., Kono, T., Yoshida, T., Sakai, M., & Itami, T. (2008). Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, Marsupenaeus japonicus. Fish and shellfish immunology, 24(1): 122 - 133.

Oka, M. (1969). Studies on Penaeus orientalis Kishinouye-VIII. Structure of the newly found lymphoid organ. Bull Jpn Soc Sci Fish, 35: 245 - 250.

Raa, J. (1996). The use of immunostimulatory substances in fish and shellfish farming. Reviews in Fisheries Science, 4(3): 229 - 288.

Ringø, E. (1993). Does dietary linoleic acid affect intestinal microflora in Arctic charr, Salvelinus alpinus (L.)? Aquaculture Research, 24(1): 133 - 135.

Sordillo, L., Shafer-Weaver, K., and DeRosa, D. (1997). Immunobiology of the mammary gland. Journal of dairy science, 80(8): 1851 - 1865.

Tanekhy, M., and Fall, J. (2015). Expression of innate immunity genes in kuruma shrimp Marsupenaeus japonicus after *in vivo* stimulation with garlic extract (allicin). Veterinarni Medicina, 60(1): 39 - 47.

Tyagi, A., Khushiramani, R., Karunasagar, I., and Karunasagar, I. (2007). Antivibrio activity of recombinant lysozyme expressed from black tiger shrimp, Penaeus monodon. Aquaculture, 272(1): 246 - 253.

Van de Braak, C., Botterblom, M., Taverne, N. v., Van Muiswinkel, W., Rombout, J., and Van der Knaap, W. (2002). The roles of haemocytes and the lymphoid organ in the clearance of injected Vibrio bacteria in Penaeus monodon shrimp. Fish & shellfish immunology, 13(4): 293 - 309.