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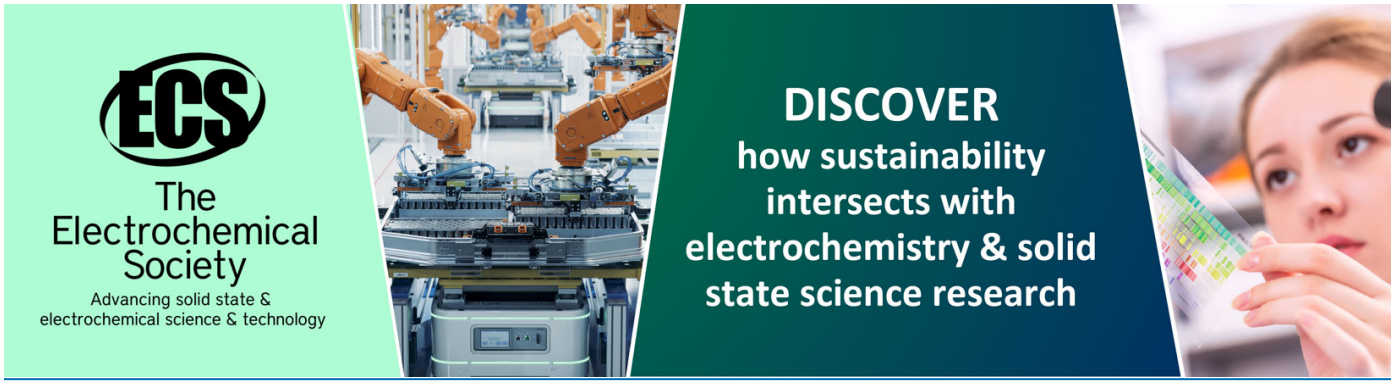

The immune responses of *Oreochromis niloticus* under different form of *Bacillus* supplementation

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Abstract. Probiotic is considered an effective means for disease prevention in the aquaculture system. The most of probiotic species used in aquaculture were *Bacillus* in the form of vegetative cells. Therefore, this study evaluates the immune responses of *Oreochromis niloticus* under the *Bacillus* supplementation in the form of vegetative cells and spore. The addition of vegetative cells and spore *Bacillus* was given in the fish diet for 49 days. Several non-specific immune responses were evaluated afterward. Treatment without *Bacillus* supplementation in the diet was used as a control. The results showed that *Bacillus* supplementation in the form of spore and vegetative increased the non-specific immune response compared to those of control. Some parameters of the immune response, such as total leukocytes, haematocrit value, and respiratory burst, were affected by the form of *Bacillus* supplementation. Total haematocrit (31.67%), total leukocytes (8,2x104 cells.mm-3), Lymphocyte (81.33%) and respiratory burst (0.09 nm) of Tilapia with spore *Bacillus* supplementation were noted as the highest value. On the other hand, the phagocytosis activity of Tilapia was found statistically similar to vegetative or spore form of *Bacillus* supplementation. The factor that affected those results was the higher viability of the *Bacillus* spore in the fish diet. This study indicated that *Bacillus* supplementation in the form of spore gave the best improvement on the Tilapia non-specific immune response and could maintain the health status of the fish.

1. Introduction

Tilapia is a well-adapted and fast-growing species. In Indonesia, tilapia production reached 1.280 million tons in 2017 [1]. In an intensive system with high-stress conditions, significant losses still have occurred due to several bacterial diseases. Motile Aeromonas Septicemia (MAS) outbreak, caused by *Aeromonas* spp, had caused economic damages up to 60% [2, 3]. This MAS disease infected tilapia in several stages of their life from juvenile, grow-out, and broodstock.

Antibiotics have been usually used to control many bacterial diseases. Yet, over-use of antibiotics to control bacterial infections has led to the emergence of multi-antibiotic resistant species[4][5]. Furthermore, antibiotic resistance in the aquaculture industry has been transmitted horizontally through gene transfer to pathogenic bacteria in humans. The use of antibiotics in animal feed has been banned [6], which then leads to the necessity for developing alternative prophylactics. Probiotics, the beneficial

bacteria, offer an alternative means to the chemotherapeutic agent in the aquaculture industry. Several studies showed that probiotics are effectively used as disease prevention agents [7]–[9].

The most well-known genus used in probiotics is *Bacillus*. These gram-positive bacteria can form endospores in their vegetative cells. Several advantages of spore over vegetative cells is toleration against toxic material, extreme temperature, desiccation, and radiation [10]. The use of bacillus spore for probiotic is more promising to produce a stable product [6], [11]. Positive results were found when *B. subtilis* probiotics in the form of spores were given to newly hatched chickens and then challenged with *Escherichia coli* [12]. In the agricultural industry, spores are an alternative to antibiotics used as growth promoters [13], [14]. *Bacillus* spores are also used for diarrheal drugs in humans, although the mechanism still cannot be explained. Even though the *Bacillus* spore had been applied for humans, agriculture, and livestock, there is still limited research of those in aquaculture. Therefore, this study aimed to evaluate the supplementation of *Bacillus* in the form of vegetative cells and spore on non-specific immune responses of Tilapia.

2. Material and methods

2.1. Bacillus Vegetative and spore supplemented diet

The bacteria used in this study were *Bacillus subtilis* UB2, which isolated from the aquaculture system. This species was confirmed morphologically, biochemically and molecularly using 16SrRNA. The culture media used in this study were yeast extract 14 $g.l^{-1}$, glucose 20 $g.l^{-1}$. Some minerals such as $MgSO_4.7H_2O$ 0.00011 g, $MnSO_4.H_2O$ 0.04 g, $FeSO_4.7H_2O$ 0.028 g, $CaCl_2.4H_2O$ 0.03 g were added for every liter of H_2O. Fermentation was conducted in an incubator shaker 100 rpm at 37 °C. The fermentation was held for 12 hours to produce vegetative cells. Meanwhile, the spore was obtained after 70 hours of fermentation and heated at 80 ºC for 15 minutes to kill the vegetative cells. Both of the bacterial cultures were centrifuged at 3,000 g for 15 min at 4 °C. The pellet was washed twice with sterile Phosphate Buffer Saline (PBS, pH 7.2) and then resuspended in PBS. Vegetative cells and spore concentration were standardized to the density of 109 $cells.ml^{-1}$. The commercial diet (32% crude protein, 6.5% crude fat, 2% ash, 7% crude fibre) was sprayed with vegetative and spore of *Bacillus* at the concentration of 109 $cells.ml^{-1}$ At a rate of 80 $ml.kg^{-1}$ feed. The sprayed feed was incubated in a sealed container for 24 hours at 35°C. The control diet was sprayed with PBS.

2.2. Experimental set up

Eight replicate groups of *Oreochromis* sp (23± 1.7 g) were assigned randomly to a determined group and fed with *Bacillus* vegetative and spore supplemented diet at the level of 5% biomass per day. The other four replicates were fed with a control diet for 49 days. Sampling was conducted every seven days to calculate the need for diets.

2.3. Hematological and non-specific immune parameters

Seven fish per experimental aquarium were anesthetized with tricaine methane sulfonate/MS 222 (0.1 ppm). Blood was drawn from caudal vein and was filled into two-third of hematocrit tubes. It then was centrifuged for 4 minutes (12,000 rpm). The readings were evaluated in the haemoctrit% value by using a scale.

Total leukocyte was performed based on the method of Noga (2000). Blood was drawn (20 µl) from caudal vein and homogenized with 4 ml Natt-Herric's stain solution (1:200). The stained blood was left at room temperature at 5 minutes and dropped to a Neubauer hemocytometer. After 5 minutes, leukocytes were counted using the 10x objective with the following formula:

$$\text{Total Leukocytes} \,.\, \text{ml}^{-1}\text{blood} = \frac{\#\ \text{leukocytes}}{8\ (\text{corner of both sides of haemocytometer}) \times 2{,}000\ (10\text{x dilution})}$$

Aliquot blood was smeared on slides with fixation in 96% methanol for 5 minutes. Staining with Giemsa was conducted after air drying at room temperature for a few minutes [16]. Slides were

examined at x1000 to evaluate the differential of leukocytes as a proportion of monocytes, neutrophils, and lymphocytes within 100 cells.

Phagocytic activity was performed following the method of [17]. Blood (0.1 ml) suspension was placed into a 96 well microtiter plate. *A. hydrophila* (0.1 ml, with the concentration of $1x10^8$ cells ml^{-1}) suspended in phosphate-buffered saline (PBS) (1:1) was added and incubated at room temperature for 30 min. A Smear from that suspension was prepared on a glass slide, fixed with 95% ethyl alcohol and stained with Giemsa for 20 min. Phagocytic activity (%) was calculated by dividing the phagocytizing cells with the number of total cells.

Respiratory Burst of Tilapia fed with and without *Bacillus* supplementation was observed based on [17]. Blood (50 ml) was placed into the wells of ''U'' bottom microtiter plates and incubated at 37 °C for one hour to allow the adhesion of cells. Then the supernatant was removed, and the wells washed three times in PBS. After washing, 50 ml of 0.2% NBT was added and incubated for a further one hour. The cells were then fixed with 100% methanol for 2-3 min and washed three times with 30% methanol. The plates were air-dried, and 60 ml 2N potassium hydroxide and 70 ml dimethyl sulphoxide were added to each well. The OD (optical density) was recorded in an ELISA reader at 540 nm.

2.4. Statistical analysis

Statistically, all data were analyzed using one-way ANOVA using SPSS 15.0. Differences among treatments were compared using Duncan's Multiple range test ($p<0.05$).

In fish, the innate immune response has been considered an essential component in combating disease incidents due to the constraints placed on the adaptive immune response by their poikilothermic nature plus the limited antibody repertoires, affinity maturation and memory and relatively slow lymphocyte proliferation [18].

3. Results and discussion

In fish, the innate immune response is essential to fight diseases as fish had many limitations on their adaptive immune system [18]. After seven weeks of culture, tilapia fed with vegetative and spore form of Bacillus showed significantly different values of the hematological condition and some nonspecific immune response parameters, as presented in Table 1.

Table 1. Non-specific immune response of Tilapia reared under different form of *Bacillus* supplementation

Parameter	Vegetative cell	Spore	Control
Total Hematocrit (%)	28.00 ± 1.00^b	31.67 ± 1.00^c	22.67 ± 0.58^a
Total Leukocyte ($x10^4$ cells.μl^{-1})	6.82 ± 2.25^b	8.28 ± 1.76^c	4.02 ± 1.26^a
Total Limphocyte (%)	79.33 ± 0.58^b	81.33 ± 0.58^c	76.22 ± 0.25^a
Total Monocyte (%)	8.33 ± 0.58^a	8.00 ± 0.58^a	9.09 ± 0.01^a
Total Neutrophile (%)	12.33 ± 0.58^b	10.33 ± 0.58^a	14.69 ± 0.25^c
Phagocytosis activity (%)	55.49 ± 1.20^b	60.16 ± 1.61^b	36.56 ± 3.59^a
Respiratory Burst (nm)	0.05 ± 0.01^a	0.09 ± 0.02^b	0.04 ± 0.00^a

Haematocrit is the ratio of red blood cells to the total body volume, which expressed with percentage. Haematocrit values found in this study were in the range of 22.67 – 31.67%, which is considered the average level in fish [19]. The result showed that the haematocrit level of Tilapia was significantly affected by the supplementation of *Bacillus* in the diet. Furthermore, it can be seen in Table 1 that the supplementation of *Bacillus* in the form of vegetative cells and spore in the diet increased the haematocrit level of Tilapia after 49 days. The highest haematocrit value was recorded in Tilapia with a spore supplemented diet (31.67%), while the lowest one was found in Tilapia with no *Bacillus* supplemented diet. In line with this finding, several studies found that the probiotic augmentation could enhance the haematocrit value in some fish such as red seabream, Tilapia, and rainbow trout [20]–[23]. The increase of haematocrit value indicated the improvement of the health status of the fish [23], [24].

Moreover, fish fed with a probiotic supplemented diet had better responses toward any stressor such as salinity stress and infection [21], [25].

Leukocytes are the most active unit of the body's defence system and circulate in the blood circulation. The primary function of leukocytes is to damage infectious and toxic materials through the process of phagocytosis. It can be seen from Figure 1 that after fed with *Bacillus* supplemented diet in the form of vegetative cells and spore, the increasing number of leukocytes was observed. The highest leukocytes number (8.28x104 cells.µl-1) was noted in Tilapia fed with a spore form of *Bacillus*. The higher production of leukocytes was also followed when applied the probiotic-supplemented diet to Tilapia [26] [27]. Other similar reports with the increase of white blood cells were also obtained in *Clarias batrachus* [28], *Epinephelus bruneus* [29], and *Cirrhinus mrigala* [30][31].

Lymphocytes are a type of leukocytes that have a spherical shape. Total lymphocytes of Tilapia increased as there were *Bacillus*-supplementation in their diet both in the form of vegetative cells and spores. The highest lymphocytes were recorded in the Tilapia with a spore supplementation diet (81.33%), while the lowest one was observed in control (76.22%). The application of *Bacillus* supplementation as a probiotic was carried out to prove the existence of cellular defence activity, which was demonstrated through increased total lymphocytes. Lymphocytes functioned as protectors against microbial agents. The increase in lymphocytes indicated cellular defence activity through antibody formation. Based on Table 1, the lymphocytes of Tilapia increased significantly, along with the supplementation of *Bacillus* in the diet.

Furthermore, the supplementation of *Bacillus* in the form of spore and vegetative cells also gave a significant effect. The highest lymphocytes were observed with supplementation of *Bacillus* spore. *Bacillus* sp. supplementation caused an increase in the percentage of lymphocytes as it factors of fish protection against microbial agents [32]. Several studies were also observed that probiotics actively stimulated the lymphocyte proliferation (B and T cells), which then increased the immunoglobulin production in fish [33]. An increase in the percent of lymphocytes leads to the rise of the resistance against the pathogen, environmental stimulation, and stress. It then decreases of mortality rate, increases the survival rate, and improves the growth.

Monocytes are the most significant type of leukocytes, which serve as the innate immune system and influence the process of adaptive immunity. Based on Table 1, there was no significant difference ($p>0.05$) of total monocytes in Tilapia blood with and without Bacillus supplementation in their diet for 49 days. The reason underlying that trend was there was no experimental infection conducted on Tilapia. Higher counts of monocytes as phagocytic cells are an indication of infection occurrence in fish [34]. Several studies recorded the increase of monocyte numbers in fish blood after it was given a probiotic supplemented diet and challenged with the pathogen. Monocyte counts of *Labeo rohita* increased after fed with *B. subtilis* supplemented the diet and infected with *Aeromonas hydrophilla* [35]. Tilapia also experienced an increase in monocytes after fed with *L. acidophilus* diet and challenged with *A. hydrophilla* [36]. In this study, however, there was a decreasing trend of monocyte count after *Bacillus* supplementation, whether in the form of *Bacillus* vegetative cells or spores. This might be since pathogen in the Tilapia digestive system reduced along with the *Bacillus* supplemented diet (vegetative cells or spore). The administration of *Bacillus* sp. also caused a reduction in the percentage of monocytes [32]. Monocytes will act as the first line of defence system when there is a foreign object enters the pathogenic bacteria.

In fish, the primary cells involved in the first stage of inflammation are neutrophils. The neutrophil may be cytokine production to acquire immune cells to the area of infection [37]. The supplementation of *Bacillus* in the form of vegetative cells or spores gave a significant effect on total neutrophil of Tilapia compared to that of control (without *Bacillus*) (Table 1). The highest neutrophil count was observed in control (14.67%), while the lowest one was noted in the *Bacillus* spore supplementation diet (10.33%). Probiotics can increase neutrophil yield as a form of the immune response to the presence of a foreign antigen or protein. Neutrophils are the first cells that leave blood vessels due to containing vacuoles that contain enzymes to destroy pathogenic organisms.

Phagocytosis is a process of pathogen internalization with the purpose of destruction, which then continued its antigen present to lymphocytes [38]. It was observed in this study that the *Bacillus* supplementation influenced the phagocytic activity of Tilapia. However, the application of *Bacillus* in

vegetative cells and spores did not have a significant effect. The highest mean of phagocytic activity (60.16%) was found in the spore treatment, while the lowest one observed in control treatment Tilapia (36.56%). The increase of phagocytic activity after *Bacillus* supplementation for 49 days were about 52-64%. Great phagocytic activity results indicated an increase in the immune system of fish. Probiotics seem to increase phagocytic activity through the production of active molecules, nitrogen, and reactive oxygen [39, 40]. The activity of phagocytosis was also noted to increase in sea bream when *Lactobacillus* and *Bacillus subtilis* were used as probiotic agents [41, 42].

Respiratory burst (RB), which is commonly called oxidative burst, is the use of reactive oxygen from various types of cells to carry out the activity of destroying foreign particles. Usually, the release of chemicals occurs from immune cells such as neutrophils and monocytes when those contact with bacteria. In this study, the RB value was significantly affected by the supplementation of *Bacillus* in the form of spore. On the other, the supplementation of *Bacillus* in the form of vegetative cells was similar to that of control. The increase of RB also observed in Nile Tilapia after probiotic supplementation with combination probiotics of *B. subtilis*, *S. cerevisiae*, and *A. oryzae* [43].

Taking in to account the results together, the significant different effect of Bacillus in the form of vegetative cells and spore on the Tilapia immune response was due to their viability when they applied on a diet. The vegetative cells of *Bacillus* experienced more death compared to *Bacillus* spore. Examination of total bacterial count showed that the viability of *Bacillus* vegetative cells on the Tilapia diet was lower (18.6%) compared to that of *Bacillus* spore (81.4%). *Bacillus*, in the form of spore, could survive in stressful environments such as dry conditions and exposure to heat [44]. *Bacillus* spore was able to survive in extreme conditions and had a more stable number compared to the vegetative one [10]. Several factors may affect probiotic efficiency, especially the probiotic and dietary dose concentration [45].

4. Conclusion

Supplementation of *B. subtilis* in the form of vegetative cells and spore influenced the non-specific immune response of Tilapia. The immune response of tilapia supplemented with *Bacillus* spore was higher than that with vegetative cells. That was due to the viability of *Bacillus* spore was higher than that of vegetative cells.

5. References

1. Pusat Data Statistik dan Informasi. Satu Data Produksi Kelautan dan Perikanan 2017 [Internet]. Kelautan dan Perikanan dalam Angka. 2018 [cited 2019 Jul 10]. Available from: https://kkp.go.id/setjen/satudata/page/1453-kelautan-dan-perikanan-dalam-angka
2. Dong HT, Taengphu S, Charoensapsri W, Khunrae P. 2017 *Fish Dis*. **40**(10)1-9
3. Wassif I 2018 *Alexandria J Vet Sci.* **57**(1)38-45
4. Andayani S, Yuniarti A 2017 *Asian Jr Microbiol Biotech Env Sci.* **19**(2)274-283
5. Holmström K, Graslund S, Wahlstrom A, Poungshompoo S, Bengtsson B-E, Kautsky N 2003 *Int J Food Sci Technol.* **38**(3)255-266
6. Hong H A, Duc L H, Cutting S 2005 *FEMS Microbiol Rev.* **29**(4)813-835
7. Mujeeb Rahiman K M, Jesmi Y, Thomas A P, Mohamed Hatha 2010 *Aquac Res.* **41**(9) e120–134
8. Selim K M and Reda R M. 2015 *Fish Shellfish Immunol.* **44,** 496-503
9. Sivakumar N, Sundararaman M, Selvakumar G 2012 *African J Biotechnol.* **11**(91): 15811-15818
10. Wolken W A M, Tramper J, Werf M J van der 2003 *Trends Biotechnol.* **21**(8): 338-345.
11. Ugoji E and Hunter C 2006 *South African J Bot.* **72,** 28-33
12. La Ragione R, Casula G, Cutting S and Woodward M 2001 *Vet Microbiol.* **79**(2): 133-142
13. Casula G and Cutting S M 2002 *App. Env.Microbiol.* **68**(5): 2344-2352
14. Paclibare JO, Usero RC, Somga JR, Visitacion R 2002 *Integration of finfish in shrimp (Penaeus monodon) culture: an elective disease prevention strategy.* In: *Disease Control in Fish and Shrimp Aquaculture in Southeast Asia Diagnosis and Husbandry Techniques. Tigbauan, Iloilo, Philippines*.: Aquaculture Department, Southeast Asian Fisheries Development Center p. 151-171

15. Edward J N 2000 *The clinical work-up*. In: *Edward J. Noga, editor. Fish Disease: Diagnosis and Treatment*. Second. Iowa: Wiley-Blackwell, A John Willey & Sons, Inc., Publication p. 13–48
16. Thrall MA 2004 *Veterinary Hematology and Clinical Chemistry ED*. Maryland, USA: Lippincott Williams and Wilkins
17. Choudhury D, Pal A K, Sahu N P, Kumar S, Das S S, Mukherjee S C 2005 *Fish Shellfish Immunol.* **19**(3): 281-291
18. Whyte S K 2007 *Fish Shellfish Immunol.* **23,** 1127-1151
19. Farrell A P 2011 *Cellular Composition of the Blood*. In: Encyclopedia of Fish Physiology: From Genome to Environment 2. p. 984–91
20. Dawood M A O, Koshio S, Ishikawa M, Yokoyama S, El Basuini M F, Hossain M S 2016 *Shellfish Immunol.* **49,** 275-285
21. Feliatra F, Elizal E, Lukistyowa I, Melina D and Ramadhani M 2017 *Asian J Anim Vet Adv.* **13**(1): 43-51
22. Garcia-Marengoni N, de Moura MC, de Oliveira NTE, Bombardelli RA, Menezes-Albuquerque D 2015 *Lat Am J Aquat Res.* **43**(3): 601–606
23. Panigrahi A, Kiron V, Kobayashi T, Puangkaew J, Satoh S and Sugita H 2004 *Vet Immunol Immunopathol.* **102**, 379e88
24. Gallaugher P 1994 *The role of haematocrit in oxygen transport and swimming in salmonid fishes*. PhD thesis
25. Imanpoor M R and Roohi Z 2015 *Turkish J Fish Aquat Sci.* **15,** 917-922
26. Jatobá A, Vieira F N, Buglione-Neto C C, Mouriño' J L P, Silva BC, Seiftter W Q 2011 *Fish Physiol Biochem.* **37**(4): 725-732
27. Aly S M, Abdel-Galil Ahmed Y, Abdel-Aziz Ghareeb A, Mohamed M F 2008 *Fish Shellfish Immunol.* **25**(1–2): 128-136
28. Dahiya T, Sihag R C, Gahlawat S K 2012 *J Fish Aquat Sci.* **7**(4): 279-290
29. Harikrishnan R, Balasundaram C and Heo M S 2010 *Fish Shellfish Immunol.* **29**(6): 1037-1043
30. Ullah A, Zuberi A, Ahmad M, Bashir Shah A, Younus N, Ullah S 2018 *Fish Shellfish Immunol.* **72**, 266-272
31. Sharma P 2013 *J Fish.* **7**(4): 323-328
32. Kamgar M, Ghane M 2014 *J Appl Environ Microbiol.* **2**(5): 203-207
33. Al-Dohail M A, Hashim R, Aliyu-Paiko M 2009 *Aquac Res.* **40**, 1642-1652
34. Mohapatra S, Chakraborty T, Kumar V, DeBoeck G, Mohanta K N 2013 *J Anim Physiol Anim Nutr* (Berl) **97**(3): 405-430
35. Behura B B, Das B K, Mukherjee SC 2015 *Int J Curr Microbiol Appl Sci.* **4**(11): 261-271
36. Aly S M, Mohamed M F, John G 2008 *Aquac Res.* 39 647-656
37. Shoemaker C A, Klesius P H, Lim C 2001. *Immunity and disease resistance in fish*. In: Lim C, Webster C D, editors. *Nutrition and Fish Health*. New York: Food Products Press; 2001. p. 176–393
38. Pohlenz C, Iii DMG 2014 *Aquaculture* **431,** 111-117
39. Adams D O, Hamilton T A 1984 *Annu Rev Immunol.* **2,** 283-318
40. Kwak J K, Park S, Koo J G, Cho M G, Buchholz R, Goetz P 2003 *Acta Biotechnol.* **23,** 359-371.
41. Picchietti S, Mazzini M, Taddei AR, Renna R, Fausto AM, Mulero V, et al 2007 *Fish Shellfish Immunol.* **22**(1) 57-67
42. Salinas I, Cuesta A, Esteban MA, Meseguer J 2005 *Fish Shellfish Immunol.* **19** 67e77.
43. Iwashita MKP, Nakandakare IB, Terhune JS, Wood T, Ranzani-Paiva MJT 2015 *Fish Shellfish Immunol.* **43,** 60-66
44. Wangka-Orm C, Deeseenthum S, Leevatcharamas V 2014 *Pakistan J Biol Sci.* **17**(8):1015-1022
45. Welker T L, Lim C 2011 *J Aquac Res Dev.* S1 014 1–8

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