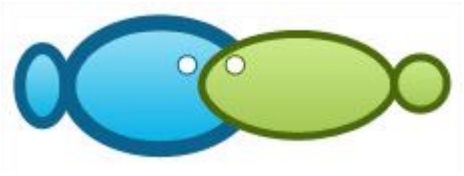

Feeding duration of dietary *Nodulisporium* sp. KT29 to prevent the infection of *Vibrio harveyi* on Pacific white shrimp *Litopenaeus vannamei*

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Abstract. This study was conducted to determine the best feeding duration in the administration of *Nodulisporium* sp. KT29 metabolites to improve the growth performance and prevent the infection of *Vibrio harveyi* on Pacific white shrimp (*Litopenaeus vannamei*) under sea floating net cages. The experiments consist of two phases, i.e 1) the preliminary test to determine the optimum dose of *Nodulisporium* sp. KT29 metabolites; 20 mL kg^{-1} and 40 mL kg^{-1} and 2) the *in vivo* test to determine the best duration of administration, this study was conducted through a completely randomized design. The treatments given before the challenge test was performed in sea floating net cage for 21 days, while the challenge test was carried out on a laboratory scale. The preliminary test results revealed that the best dose of *Nodulisporium* sp. KT29 used for in vivo test was 20 mL kg^{-1} of feed. The result of in vivo test showed that the administration of *Nodulisporium* sp. KT29 metabolites for seven days resulted the best growth performance, survival rate and immune response after the challenge test.
Key Words: antibacterial, immunostimulant, marine fungal metabolites, immune response.

Introduction. Aquaculture is one of the fisheries sectors providing world production of fish to supply the demand of protein source for human. By 2014, Food and Agriculture Organization (FAO) estimated that aquaculture had become a larger source for food than wild capture (Asche 2015). The contribution of aquaculture in total fish supply grew from 9% in 1980 to 48% in 2011 (FAO 2013). Globally, aquaculture activity grew quickly by producing crustaceans, which recorded a production value of 6,446,818 tons in 2012 (FAO 2014), with shrimp as the main species. Indonesia has been the fourth-largest marine shrimp farming sub-sector in the world, which had a total production of 547,934 tons in 2012. Pacific white shrimp (*Litopenaeus vannamei*) is the most populous species cultivated in Indonesia, accounting for 46% of national production (Portley 2016), which primarily depends on coastal brackish-water ponds. On the other hand, the shrimp culture in brackish-water ponds has several negative impact, such as the destruction of mangroves due to the need to open a space in mangrove zones, marsh, and coastal lagoons, the water pollution caused by nutrients, organic matter, and sediments, the negative impact from the use of antibiotics and other chemical substances contaminated water reservoir/the shrimp and disease outbreaks (Primavera 1998). The shrimp farming practice in open sea using floating net cages is one of innovative shrimp culture technologies, which has several advantages compared to the shrimp culture in brackish-water ponds; it provides the higher water flow rate that removes toxic nitrogenous metabolites and provides oxygen, it requires a lower production cost, because it does not need an energy source for aeration/water exchange (Paquotte et al 1998), it has a high possibility for intensification due to the high carrying capacity of sea water body, it produces a better feed conversion ratio due to the availability of sufficient natural feed as nutrient resource for the cultured species (Zarain-Herzberg et al 2010), and there is no

accumulation of solid water near the cages (Alongi et al 2003). However, the shrimp cultivation using sea floating net cages has several problems, such as dynamic properties of sea water, which are strongly influenced by environmental factors, such as waves, currents, turbidity, and tides (Azis 2006). This method is also prone to be disrupted with changes in environmental conditions and the expansion of predators that can lead to stress/diseases caused by physiological stress on the cultivated organism (Beveridge 1984; Swann et al 1994). The stress on the shrimp will lead to an increasing use of energy for the organism defense system which will cause a decreasing in the shrimp production performance, such as the reduction on growth and feed utilization (Peterson & Walker 2002).

Vibriosis is a bacterial disease that often attacks the shrimp with *Vibrio harveyi* as the pathogenic agent (Widanarni et al 2012). This pathogen is more virulent when the shrimp body condition decreases (Austin & Austin 1999). When the outbreak happens, the population of this pathogen may increase to thousand times, causing the mortality rate up to 100% (Lightner 1983). The prevention of bacterial diseases commonly uses antibiotics as the prevention agent, but now the use of antibiotics has been prohibited, because it can lead the emergence of bacterial resistance species (Rhodes et al 2000) and it requires a long time for antibiotic residues withdrawal from the fish body (Esposito et al 2007). The presence of antibiotics in water and sediment can affect normal flora, plankton, and animals, which can induce changes in microbiota diversity and ecological balance (Cabello 2006).

One of alternative approaches that can be applied to improve the production performance and prevent bacterial infection is through the use of natural materials. The use of natural materials has several advantages, such as it is safe and environmentally friendly. One of the natural materials that can be used is a marine fungus, which generally contains β-glucan and antibacterial compounds. *Nodulisporium* sp. KT29 is a fungus isolated from the marine red alga (Tarman et al 2011a). This marine fungus has antibacterial compounds to inhibit bacterial pathogens attacking aquatic organisms, such as *Vibrio anguillarum*, *Aeromonas salmonicida*, and *Yersinia ruckeri* (Tarman et al 2011b). According to Achmadi (2015), the use of marine fungi as the feed fermenter could improve the growth performance and the production of red blood cells on catfish (*Clarias* sp.). The use of *Nodulisporium* sp. KT29 metabolites on *L. vannamei* cultivated using sea floating net cages has not been studied. Therefore, this study was conducted to determine the best duration in the administration of *Nodulisporium* sp. KT29 metabolites to improve the growth performance and prevent the infection of *V. harveyi* on Pacific white shrimp cultured using sea floating net cages.

Material and Method. This study was carried out for eight months started from March to October 2015. This study was held at Aquatic Products Microbiology Laboratory, Department of Aquatic Products Technology, Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Sea Floating Net Cage of Sea Farming Center, Center for Coastal and Marine Resources Studies, Bogor Agricultural University, Thousand Islands, Jakarta.

Bacterial isolate used in this study was *V. harveyi* obtained from the Fish Health Laboratory collection, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University. The post larvae (PL) 12 of *L. vannamei* were derived from a private hatchery in Anyer, West Java. Marine fungal isolate (*Nodulisporium* sp. KT29) is a collection of Aquatic Products Microbiology Laboratory, Department of Aquatic Products Technology, Faculty of Fisheries and Marine Science, Bogor Agricultural University.

The preparation of Nodulisporium sp. KT29 metabolites. The rejuvenation of *Nodulisporium* sp. KT29 isolate was done on Potato Dextrose Agar (PDA) for seven days. The rejuvenated isolate was then cut in the shape of a cube for pre-culture in 250 mL Erlenmeyer flask that had been filled with 100 mL Potato Dextrose Broth (PDB). The pre-culture was carried out for seven days. After that, 12.5 mL *Nodulisporium* sp. KT29 culture was taken and was transferred to a 500 mL Erlenmeyer flask filled with 250 mL

PDB medium. This mixture was cultured for 14 days. The result of this culture step was harvested and was filtered using Whatmann filter paper to separate the mycelia and culture broth. The part used for the study was the culture broth containing metabolites of the fungus *Nodulisporium* sp. KT29. The culture broth used for *in vitro* test was evaporated to separate the moisture content contained in the metabolites. The evaporated metabolites were then added to the feed. *Nodulisporium* sp. KT29 metabolites contained bioactive compounds including 0.54% β-(1,3) glucan and phytochemical compounds (121 ppm phytosterol, 23 ppm saponin and 31 ppm polyphenol) (Saputra et al 2016).

The manufacture of experimental feed. There were two experimental feed used in this study; the commercial feed and the treatment feed. Commercial feed was a regular feed used in shrimp farming. Commercial feed (36-35% protein content) was re-pelleted and was added with 0.1% vitamin C + CMC (carboxyl methyl cellulose) as a binder at an amount of 30 g kg^{-1} of feed. The treatment feed was a commercial feed, which was re-pelleted and was added with 0.1% vitamin C, CMC 30 g kg^{-1} of feed, and *Nodulisporium* sp. KT29 metabolites. There were two doses of *Nodulisporium* sp. KT29 metabolites used in this study; 20 mL kg^{-1} of feed (P1) and 40 mL kg^{-1} of feed (P2). After re-pelleting process, the experimental feed was mashed into a crumble.

Experimental design. The study about the administration duration, was carried out after the optimum dose obtained during in vitro test and the preliminary study, in this case the doses tested were 20 mL kg^{-1} and 40 mL kg^{-1}. The method used was an experimental method, through a completely randomized design (CRD). The treatments given before the challenge test was performed in sea floating net cage for 21 days, those consisted of three treatments with three replicates including the control/commercial feed (K), the administration of treatment feed for seven days and then challenged by *V. harveyi* (P7), the administration of treatment feed for 14 days and then challenged by *V. harveyi* (P14). The challenge test was carried out on a laboratory scale through immersion method using *V. harveyi* suspension at a concentration of 10^6 CFU mL^{-1} according to the result of LC_{50} test. After the challenge test, the treatments given consisted of six treatments with three replicates including the positive control/the immersion in *V. harveyi* suspension (KP), the negative control/the immersion in physiological solution (KN), the administration of treatment feed for seven days and then challenged by *V. harveyi* (P7+), the administration of treatment feed for seven days and without the challenge test (P7-), the administration of treatment feed for 14 days and then challenged by *V. harveyi* (P14+), and the administration of treatment feed for 14 days and without the challenge test (P14-). After the experimental infection, the shrimps were reared for 10 days.

Culture condition. The rearing medium used for rearing of shrimp on a field scale was the floating net cage (3x3x0.3 m^3) with a partition net (1x1x2.5 m^3) to rear *L. vannamei* from each treatment. Shrimps were acclimatized for a day after stocked into the floating net cage. The stocking density of the shrimp was 700 shrimps per m^3. Feed was given with a feeding rate of 50-35% three times a day (07.00 am, 12.00 am and 05.00 pm).

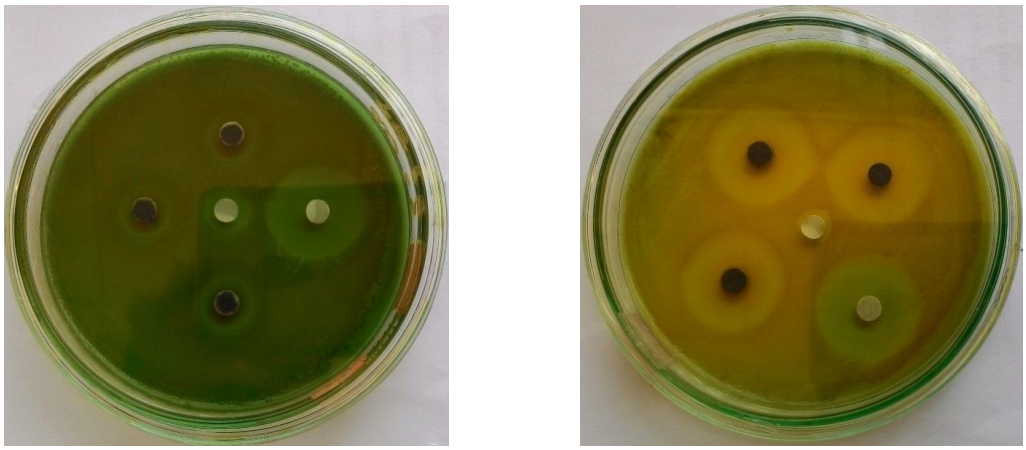
The rearing medium used for the challenge test was the aquarium (30x20x20 cm^3) located in Fish Health Laboratory, Bogor Agricultural University. Shrimps were adapted in the aquarium for three days before being challenged by *V. harveyi*. The stocking density of the shrimp in this phase was 20 shrimps per aquarium. The feed was given with a feeding rate of 15% three times a day (07.00 AM, 12.00 AM and 05.00 PM).

Experimental parameters. Experimental parameters observed at in vivo test included growth performance parameters (daily growth rate and feed conversion ratio), immune response parameters (phenoloxidase activity, respiratory burst activity, survival rate), and stress response parameter (the glucose content in the hemolymph).

Statistical analysis. Data were tabulated using MS. Office Excel 2010 and were tested with ANOVA using SPSS 16 with a 95% confidence interval. The treatments that showed significantly different results were tested by Duncan test to determine the best treatment. Immune response parameters, growth performance and stress response were presented in tables and graphs.

Results and Discussion

In vitro test. The doses of *Nodulisporium* sp. KT29 metabolites used for in vitro test were 10 and 20 µL according to Saputra (2016). The result of in vitro test showed that *Nodulisporium* sp. KT29 metabolites had antibacterial activity against *V. harveyi*. The administration of *Nodulisporium* sp. KT29 metabolites at a concentration of 20 µL produced an inhibition zone with a radius length of 12 mm, which was equal to the radius length of inhibition zone formed by the positive control (30 µL oxytetracycline), while the administration of *Nodulisporium* sp. KT29 metabolites at a concentration of 10 µL only produced an inhibition zone radius with a length of 7 mm (Figure 1a, b). From the result of in vitro test, it could be known that the best dose to inhibit the growth of *V. harveyi* was 20 µL, which was converted to 20 mL (culture broth). This dose would be the basis of the doses used in the preliminary test.



A B

Figure 1. Inhibition zone formed on each dose of *Nodulisporium* sp. KT29 metabolites. A: 10 µL (black circles), negative control (the white circle in the middle), positive control (the white circle at the left side of negative control); B: 20 µL (black circles), negative control (the white circle in the middle), positive control (the white circle at the right side of the black circle located in the bottom) (original).

Nodulisporium sp. KT29 is an endophytic fungus, which lives in healthy plant tissues at certain or all stages of its life cycle without affecting the plant significantly (Zhang et al 2012). Endophytic fungi are known to produce bioactive compounds that have antimicrobial and antioxidant activity (Gunatilaka 2006; Jia et al 2016). The bioactive components are generally contained in the endophytic fungal metabolites. Dai et al (2006) reported that the crude ethyl acetate extract of *Nodulisporium* sp. (internal strain No. 7080) showed antifungal, antibacterial, and algicidal activities. Based on the analysis of bioactive components in *Nodulisporium* sp. KT29 metabolites, it was known that *Nodulisporium* sp. KT29 metabolites contained β-glucans, phytosterols, saponins and polyphenols. The inhibition zones of *Nodulisporium* sp. KT29 against *V. harveyi* were formed due to the presence of saponins in *Nodulisporium* sp KT29 metabolites. Saponins generally act as antibacterial substances (Citarasu 2010), because they exert some antibacterial activity by combining with cell membranes to elicit changes in cell morphology leading to cell lysis (Moyo et al 2012).

Preliminary test. The parameters observed in the preliminary test were survival rate and weight gain of *L. vannamei* fed *Nodulisporium* sp. KT29 metabolites at different

doses. The preliminary test was the toxicity test of feed containing *Nodulisporium* sp. KT29 metabolites at different doses to determine the optimum dose of *Nodulisporium* sp. KT29 added into the shrimp feed, because the effects of compounds contained in endophytic fungal metabolites are characterized by a dose-related effect, the exceeding supplies may result into abnormal growth (Nicoletti & Fiorentino 2015). The preliminary test result showed that survival rate and weight gain of *L. vannamei* fed *Nodulisporium* sp. KT29 metabolites were not significantly different ($P>0.05$) with the control (97.78±3.85%; 0.26±0.04 g). Survival rate and weight of *L. vannamei* in the treatment of the administration of *Nodulisporium* sp. KT29 metabolites at a dose of 20 mL kg^{-1} of feed (80.00±34.64%; 0.35±0.11 g) were also not significantly different ($P>0.05$) compared to a dose of 40 mL kg^{-1} of feed (84.44±18.95%; 0.40±0.14 g) (Table 1). This indicated that the feed-containing *Nodulisporium* sp. KT29 metabolites at doses of 20 mL kg^{-1} of feed and 40 mL kg^{-1} of feed were not toxic and safe for *L. vannamei*. From the preliminary test result, it could be known that the dose was used for in vivo test was 20 mL kg^{-1} of feed.

Table 1

Survival rate and weight gain of *Litopenaeus vannamei* administered *Nodulisporium* sp. KT29 at different doses (preliminary test)

Parameter	*Treatment*		
	Control	*P1*	*P2*
Survival rate (%)	97.78±3.85[a]	80.00±34.64[a]	84.44±18.95[a]
Weight gain (g)	0.25±0.04[a]	0.35±0.11[a]	0.40±0.14[a]

Different superscript letters in the same row indicate significant different results ($P<0.05$).

In vivo test. The administration of *Nodulisporium* sp. KT29 metabolites for 7 days (P7) generated the highest survival rate value of *L. vannamei* (65.3±5.86%), that was significantly different ($P<0.05$) from the control. The administration of *Nodulisporium* sp. KT29 metabolites also provided the higher daily growth rate values ($P<0.05$) compared to the control, followed by the lower feed conversion values ($P<0.05$) compared to the control (Table 2).

Table 2

Survival rate, daily growth rate and feed conversion ratio of *Litopenaeus vannamei* after administered *Nodulisporium* sp. KT29 metabolites at different durations of administration (before the challenge test)

Parameter	*Treatment*		
	Control	*P7*	*P14*
Survival rate (%)	55.9±1.41[a]	65.3±5.86[b]	61.3±4.47[ab]
Daily growth rate (% day^{-1})	16.51±0.48[a]	19.74±0.23[b]	19.89±0.72[b]
Feed conversion ratio	6.11±0.31[a]	3.46±0.12[b]	3.44±0.54[b]

Different superscript letters in the same row indicate significant different results ($P<0.05$).

The better survival rate, daily growth rate, and feed conversion ratio in shrimps treated by the administration of *Nodulisporium* sp. KT29 were supposed due to the presence of phytochemical substances, which played the role as immunostimulants and antioxidants (β-glucans and polyphenols); β-glucans are the most commonly applied immunostimulants in aquaculture (Soltanian et al 2009; Kiron 2012), which have been extensively used to reduce the negative effects of stress, increase diseases resistance, and improve various physiological performance (growth and feed conversion ratio) (Cook et al 2003; Cain et al 2003; Shelby et al 2007; Welker et al 2007). Polyphenols are secondary metabolites of plants, which have the role as antioxidants, which may protect cell constituents against oxidative damage and limit the risk of various degenerative diseases associated to oxidative stress (Pandey & Rizvi 2009; Scalbert et al 2005). According to Hai & Fotedar (2009), the administration of β-glucan on shrimp caused the

structure of intestinal surface becoming wider, so that the nutrients absorption became better. The improvement on the digestion and the nutrients absorption will cause the enhanced feed efficiency and protein absorption, which will generate a higher growth performance (Dawood et al 2015). This happens, because β-glucans entering the digestive gland will be degraded by glucanase for the energy production, causing the use of more protein for feed utilization and growth (López et al 2003). The better feed conversion ratio values on shrimps treated with the administration of *Nodulisporium* sp. KT29 metabolites were supposed due to the presence of phytochemicals contained in *Nodulisporium* sp. KT29 metabolites (phytosterols and saponins), which made the feed utilization being more efficient. According to Couto et al (2014), the administration of phytosterols and saponins, either separately or together, in juvenile gilthead sea bream (*Sparus aurata*) could improve the feed utilization. The positive effect of β-glucans, particularly in growth, is dependent on the amount of β-glucans in the diet, duration of feeding, environmental temperature, and the species under study (Dalmo & Bøgwald 2008). From the results of this study, it could be known that the administration of *Nodulisporium* sp. KT29 for seven days had better results in the growth performance of shrimps.

The administration of *Nodulisporium* sp. KT29 to *L. vannamei* under sea floating net cages was proved to be able to produce the better survival rate values after challenge test ($P<0.05$) compared to the positive control (KP). The highest survival rate of *L. vannamei* challenged with *V. harveyi* obtained in P14+ (91.67±2.89%), that were significantly different ($P<0.05$) from KP (43.33±2.88%), but it was not significantly different ($P>0.05$) from P7+ (90±10.0%) (Figure 2).

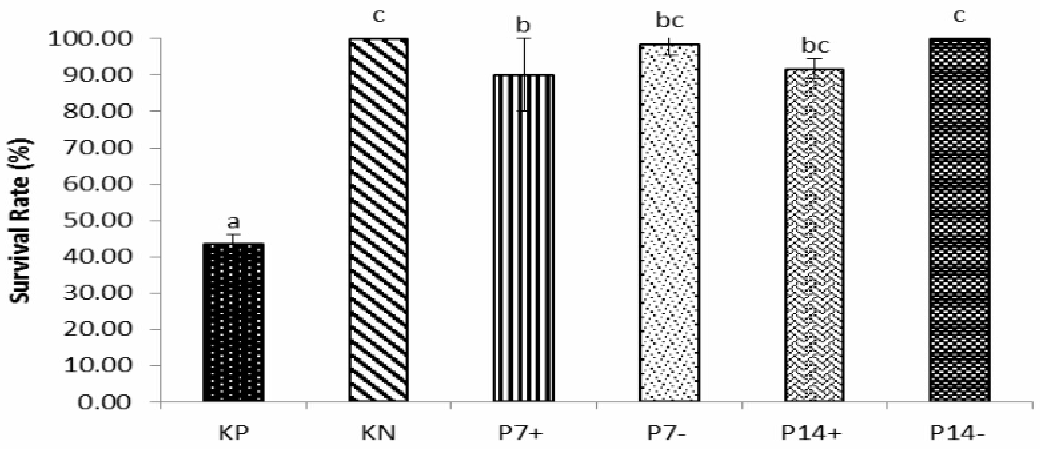


Figure 2. Survival rate of *Litopenaeus vannamei* after the challenge test. Different letters on each bar indicate significant different results ($P<0.05$).

The better survival rate after the challenge test on shrimps treated by the administration of *Nodulisporium* sp. KT29 was caused by the presence of immunostimulant, antioxidant, and antibacterial substances contained in *Nodulisporium* sp. KT29. According to Sakai (1999), glucans have immunostimulatory effects for fish and shrimp by enhancing phagocytic activity (Yano et al 1989; Chen & Ainsworth 1992; Jørgensen et al 1993). Sung et al (1994) reported that tiger shrimp (*Penaeus monodon*) immersed in yeast glucan solution (0.5 and 1 mg mL^{-1}) showed an enhancing protection against the infection of *Vibrio vulnificus*. Saponins contained in *Nodulisporium* sp. KT29 had a function as antibacterial substances, which inhibit the growth of *V. harveyi* infecting shrimps. Some saponins have also been found to have anti oxidative or reductive activity (Francis et al 2002). On the other hand, the duration of administration affects the efficacy of bioactive compounds. Couso et al (2003) reported that fish fed 10 g kg^{-1} glucans for two weeks had no protection from the disease caused by *Photobacterium damselae*, but when it was given for a week, glucans were effective in reducing disease

incidence. A decreasing of the immunostimulant efficacy in long-term administration may be due to negative feedback systems against immunostimulation, which cause the immune response reverting to the previous state (Sakai 1999). From the result of this study, it could be recommended that *Nodulisporium* sp. KT29 metabolites will be more effective to improve disease resistance of Pacific white shrimp against *V. harveyi*, when it was administered for seven days.

The feeding of the feed containing *Nodulisporium* sp. KT29 metabolites with different durations of administration showed no significantly different results ($P>0.05$) on phenoloxidase activity of Pacific white shrimp before the challenge test (day 25) among treatments. The effect of feeding of the feed containing *Nodulisporium* sp. KT29 metabolites with different durations on phenoloxidase occurred after the challenge test (day 27, 29 and 31). Phenoloxidase activity of Pacific white shrimp increased after the challenge test. The highest phenoloxidase activity values after the challenge test (day 27 and 29) were obtained at P7+ (0.243±0.005; 0.252±0.003 OD 492 nm), that were significantly different ($P<0.05$) with other treatments. The peak of the increasing of phenoloxidase activity occurred on day 31, with the highest value obtained in P14+ (0.269±0.003 OD 492 nm) followed by P7+, KN, P7-, P14-, and KP (0.253±0.004; 0.226±0.002; 0.220±0.002; 0.218±0.002; 0.156±0.004 OD 492 nm) (Figure 3).

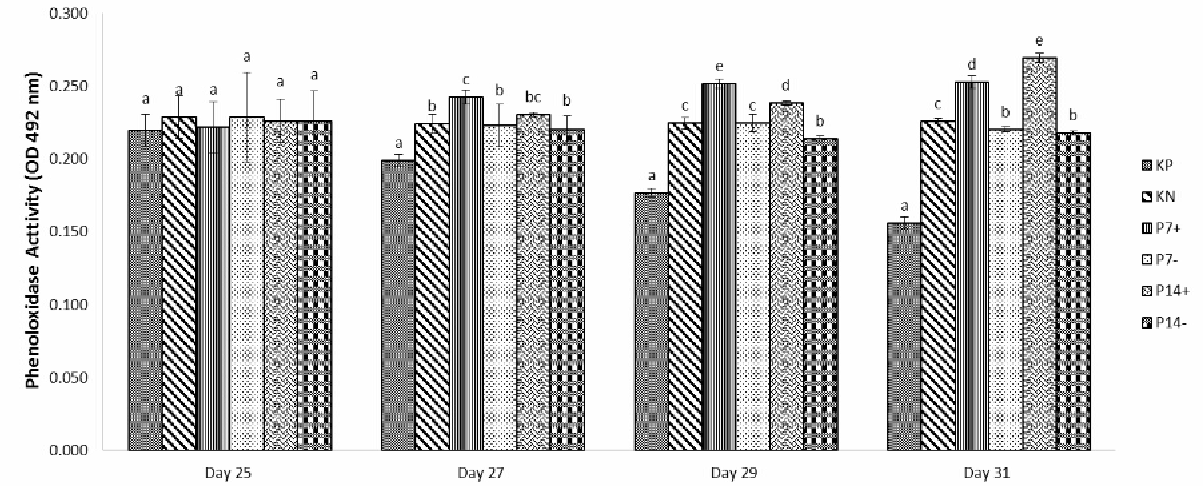


Figure 3. Phenoloxidase activity of *Litopenaeus vannamei* administered *Nodulisporium* sp. KT29. Different letters on the same day indicate significant different results ($P<0.05$).

The phenoloxidase activity is an important component in fighting microbial infections (Vargas-Albores & Yepiz-Plascencia 2000; Chiu et al 2007). Phenoloxidase is an enzyme that is responsible for the melanization process on crustaceans as a response to foreign invaders (Sritunyalucksana & Söderhäll 2000), so in the present study, phenoloxidase activity of the experimental shrimps increased after the challenge test as a response of the infection. In the haemolymph, it has a function as an inactive pro-enzyme or also known as proPO. Transformation from proPO to PO involves several reactions in the proPO activing system (Rodríguez & Le Moullac 2000). The ProPO system can be activated by immunostimulants, such as β-glucan and lipopolysaccharide (LPS) (Smith et al 2003). This was the reason of the better values of phenoloxidase activity of the experimental shrimps (treated with *Nodulisporium* sp. KT29) after the challenge test. This study found that phenoloxidase activity had still increased until the end of the study (day 31/6 days after the challenge test). This associated with the incubation period of *V. harveyi* in the shrimp body. Munaeni et al (2014) reported that phenoloxidase activity of the Pacific white shrimp challenged by *V. harveyi* increased until the end of the study (day 40/9 days after the challenge test).

The feeding of the feed containing *Nodulisporium* sp. KT29 metabolites with different durations of administration also did not show significantly different results ($P>0.05$) on respiratory burst activity of *L. vannamei* before the challenge test (day 25) among treatments. The effect of the feeding of the feed containing *Nodulisporium* sp.

KT29 metabolites with different durations on respiratory burst activity occurred after the challenge test (day 27, 29 and 31). Respiratory burst activity of *L. vannamei* increased after the challenge test with the peak occurred on day 31. Respiratory burst activity after the challenge test (day 27, 29 and 31) on P7+ (0.441±0.016; 0.472±0.021; 0.571±0.002 OD 630 nm) and P14+ (0.443±0.013; 0.482±0.013; 0.575±0.004 OD 630 nm) were higher ($P<0.05$) compared to KN, KP, P7-, and P14- (Figure 4).

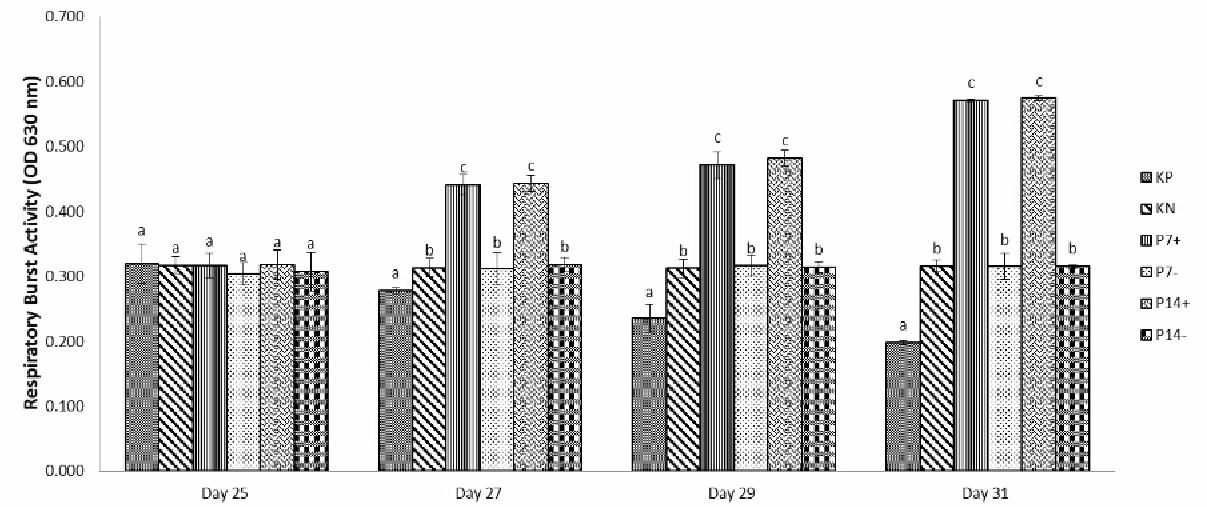


Figure 4. Respiratory burst activity of *Litopenaeus vannamei* administered *Nodulisporium* sp. KT29 metabolites on pre-challenge period (day 25) and post-challenge period (day 27, 29 and 31). Different letters on the same day indicate significant different results ($P<0.05$).

Rodríguez & Le Moullac (2000) describe respiratory burst as a mechanism for elimination of particles by phagocytic cells, which involves the release of degradative enzymes to the phagosome (oxygen-dependent killing mechanism). The present study recorded that respiratory burst activity of *L. vannamei* increased after the challenge test. The increasing in respiratory burst could be associated with the increasing of phagocytic cells activity (Rawling et al 2012) induced by an infection. According to Rieger & Barreda (2011) respiratory burst will increase oxygen consumption, which will cause the formation of superoxide anion, which is accelerated by NADPH-oxidase and multi-component enzyme contained inside the plasma membrane after the activation of phagocytic cells. The high values of respiratory burst activity in the shrimps treated with *Nodulisporium* sp. KT29 metabolites were caused by the presence of immunostimulant or β-glucan in *Nodulisporium* sp. KT29 metabolites. The study of Sarlin & Philip (2011) showed that the administration of marine yeast could enhance respiratory burst activity on *Fenneropenaeus indicus*, marine yeast is an immunostimulant source, which contains β-glucans and is potential to be applied in aquaculture practices.

Before the challenge test (day 25), the values of glucose level in hemolymph of *L. vannamei* fed the feed containing *Nodulisporium* sp. KT29 metabolites for 7 days (P7 +) and 14 days (P14-) were not significantly different ($P>0.05$) with KN and KP. However, glucose levels in hemolymph of *L. vannamei* increased after the challenge test (day 27, 29 and 31). The peak of glucose levels in hemolymph occurred on day 31 with the highest value obtained in KN (157.71±13.69 mg dL^{-1}), that was significantly different ($P<0.05$) with all treatments. After the challenge test, the *L. vannamei* challenged with *V. harveyi* (KP, P7+ and P14+) had higher glucose levels in hemolymph ($P<0.05$) compared to *L. vannamei*, which was not challenged with *V. harveyi* (KN, P7-, and P14-) (Figure 5).

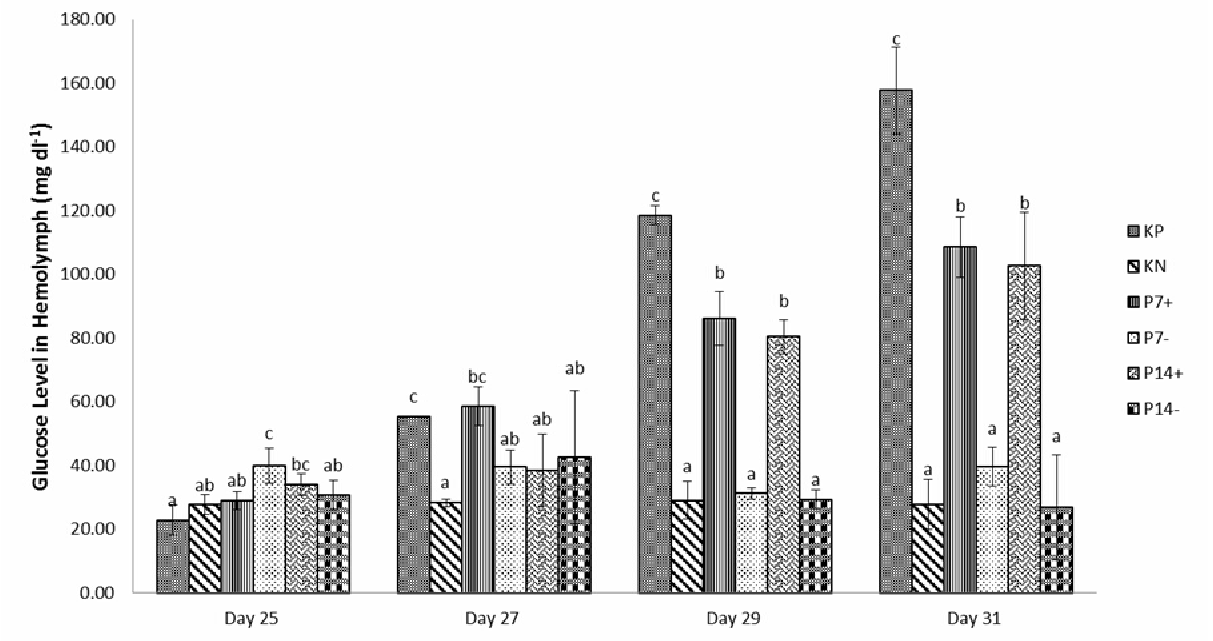


Figure 5. Glucose level in hemolymph of *Litopenaeus vannamei* administered *Nodulisporium* sp. KT29 metabolites on pre-challenge period (day 25) and post-challenge period (day 27, 29 and 31). Different letters on the same day indicate significant different results ($P<0.05$).

The increase of glucose levels after the challenge test indicated that there was the large energy requirement due to the entry of pathogen into the shrimp body. According to Lorenzon et al (2007), the glucose concentration in the hemolymph increases as a response to a stress caused by a disease. If glucose level continues to increase, it can cause the decrease of immune response of the shrimps. According to Verghese et al (2007), stress can cause a decline in immunological ability against a disease. The administration of *Nodulisporium* sp. KT29 metabolites showed a positive result on the glucose level of shrimps. It was caused by the antioxidants contained in *Nodulisporium* sp. KT29 metabolites. Polyphenols and saponins are known as antioxidant substances (Pandey & Rizvi 2009; Akinpelu et al 2014), which protect cells against the damaging effects of reactive oxygen species and free radicals, which result in oxidative stress (Mattson & Cheng 2006). Antioxidants facilitate the body natural defense mechanisms against the damaging effects of free radicals and oxidation reactions that damage cells and cause a disease. The main function of antioxidants is to prevent oxidation in various ways. Some antioxidants play a very important role in the body by preventing the oxidative damage, particularly oxidative DNA damage (Akinpelu et al 2014).

Conclusions. The result of in vivo test showed that the administration of *Nodulisporium* sp. KT29 metabolites for seven days resulted the best growth performance, survival rate and immune response after the challenge test.

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