

Supplementation of *Nodulisporium* sp. KT29 induced by *Vibrio harveyi* as an immunostimulant for controlling vibriosis in vannamei white shrimp under marine culture system

Suplementasi *Nodulisporium* sp. KT29 yang diinduksi *Vibrio harveyi* sebagai imunostimulan terhadap pengendalian vibriosis pada udang vaname yang dibudidayakan di laut

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ABSTRACT

The purpose of this study is to evaluate the effectiveness of *Nodulisporium* sp. KT29 supplementation with various *Vibrio harveyi* induction in feed against vibriosis in vannamei white shrimp. The study design included KP (positive control without supplementation *Nodulisporium* sp. KT29 and infected with *V. harveyi*), KN (negative controls without *Nodulisporium* sp. KT29 and infected with physiological solution), NT (treatment of supplementation *Nodulisporium* sp. KT29 20 mL/kg and infected with *V. harveyi*), NM (treatment of supplementation test *Nodulisporium* sp. KT29 induction of *V. harveyi* dead cell 20 mL/kg and infected *V. harveyi*), NH (treatment of supplementation *Nodulisporium* sp. KT29 induction of *V. harveyi* live cell 20 mL/kg and infected with *V. harveyi*). The study parameters included inhibition zone, resistance, immune responses, and hemolymph glucose. The results showed *Nodulisporium* sp. KT29 with induction treatment raised antibacterial activity with best treatment of NM and NH ($P < 0.05$). The results of *V. harveyi* infection resistance presented NM treatment of 20 mL/kg increase survival in vannamei shrimp reached 72.2% ($P < 0.05$). In addition, the same treatment increase the immune response activity and decrease the activity of hemolymph glucose. It could be concluded that providing NM 20 mL treatment boosted the resistance and the immune system in vaname shrimp to control vibriosis reared at the sea.

Keywords: antibacterial, β -glucan, induced, *Nodulisporium* sp. KT29, *Vibrio harveyi*

ABSTRAK

Tujuan penelitian yaitu mengevaluasi efektivitas suplementasi *Nodulisporium* sp. KT29 dengan berbagai perlakuan induksi *Vibrio harveyi* dalam pakan terhadap pengendalian vibriosis pada udang vaname yang dibudidayakan di laut. Rancangan penelitian meliputi KP (kontrol positif tanpa suplementasi *Nodulisporium* sp. KT29 dan diinfeksi *V. harveyi*), KN (kontrol negatif tanpa *Nodulisporium* sp. KT29 dan diinfeksi larutan fisiologis), NT (perlakuan uji suplementasi *Nodulisporium* sp. KT29 20 mL/kg dan diinfeksi *V. harveyi*), NM (perlakuan uji suplementasi *Nodulisporium* sp. KT29 diinduksi sel mati *V. harveyi* 20 mL/kg dan diinfeksi *V. harveyi*), NH (perlakuan uji suplementasi *Nodulisporium* sp. KT29 induksi sel hidup *V. harveyi* 20 mL/kg dan diinfeksi *V. harveyi*). Parameter penelitian meliputi zona hambat, resistensi, respons imun, dan glukosa hemolimf. Hasil penelitian menunjukkan *Nodulisporium* sp. KT29 dengan perlakuan induksi dapat meningkatkan aktivitas antibakteri dengan perlakuan terbaik NM dan NH ($P < 0.05$). Hasil pengamatan resistensi infeksi *V. harveyi* menunjukkan perlakuan NM 20 mL/kg dapat meningkatkan kelangsungan hidup pada udang vaname mencapai 72.2% ($P < 0.05$). Perlakuan yang sama juga meningkatkan respons imun dan menurunkan aktivitas glukosa hemolimf. Disimpulkan bahwa pemberian NM 20 mL dapat meningkatkan resistensi dan sistem imun udang vaname terhadap pengendalian vibriosis di laut.

Kata kunci : antibakteri, β -glucan, induksi, *Nodulisporium* sp. KT29, *Vibrio harveyi*,

INTRODUCTION

The white shrimp *Litopenaeus vannamei* is a major commodity in Indonesia. The production of white shrimp is increasing each year to fulfill market demand. Several issues during white shrimp rearing are required to be eliminated, such as water quality, nutrition, biosecurity, and disease (Ekasari *et al.*, 2016). The disease control becomes main priority in raising white shrimp production. One of the most frequently disease which attacked white shrimp is vibriosis caused by *Vibrio harveyi* (Widanarni *et al.*, 2012). The most common ways to control the disease are using antibiotics (Defoirdt *et al.*, 2011), SPF juvenile selection (Lightner, 2005), using normal ingredients, and sinbiotics which acts as immunostimulant (Sirirustananun *et al.*, 2011; Zubaidah *et al.*, 2015). The antibiotics application was prohibited nowadays because of the food security issue. It is confirmed that the requirement of natural ingredients to control disease outbreak is urgently needed. Therefore, immunostimulant is one of the environmental friendly alternative in boosting immune system towards bacteria.

Nodulisporium sp. KT29 is endophyte mold isolated from red algae *Euchema edule*. It contains antibacterial and antioxidant compounds, such as β -glukan, saponin, polyphenol, and phytosterol (Tarman *et al.*, 2011). The application of *Nodulisporium* sp. KT29 as immunostimulant was known to enhance shrimp immune system towards vibriosis (Wahjuningrum *et al.*, 2016). The high dosage utilization of *Nodulisporium* sp. KT29 is considered as high cost production, so that further evaluations were required to reduce production cost.

The utilization of natural ingredients to increase the eco-friendly active ingredients can be done using bacteria induction, salinity, pressure, light, and temperature (Tarman *et al.*, 2011). The bacteria induction method was applied in this study with natural ingredients *Nodulisporium* sp. KT29. The enhancing of active ingredients in *Nodulisporium* sp. KT29 was conducted using *Vibrio harveyi* induction to produce secondary metabolite. Mearns-Spragg *et al.* (1998) reported that the addition of pathogenic bacteria, such as *Escherichia coli* which collectively cultured with *Fucus vesiculosus* algae was able to boost secondary metabolite, such as saponin. The secondary metabolite itself was used to induce shrimp immune system towards vibriosis. This study aimed to evaluate the application of

Nodulisporium sp. KT29 metabolite induced by *Vibrio harveyi* to elevate the resistance and immune system of white shrimp towards vibriosis.

MATERIALS AND METHODS

Location

This study was conducted in August 2016 until March 2017. It took place in Fish Health Laboratory, Aquaculture Department, Microbiology of Aquatic Products Laboratory, Aquatic Product Technology Department, Faculty of Fisheries and Marine Sciences, IPB University and sea farming PKSPL LPPM-IPB, Semak Daun, Thousands Island, Jakarta.

Experimental materials

The experimental materials were white shrimp PL₁₀, *Nodulisporium* sp. KT29 isolate, and *Vibrio harveyi*. The average weight and length of white shrimp was 0.014 ± 0.01 g and 0.8 ± 0.01 cm, respectively. The post larvae were produced by PT. Suri Tani Pemuka, Anyer, Banten, Indonesia. The *Nodulisporium* sp. KT29 isolate was got from Microbiology of Aquatic Products Laboratory, Aquatic Product Technology Department, while the *Vibrio harveyi* isolate was picked from Fish Health Laboratory, Aquaculture Department, IPB University.

Nodulisporium sp. KT29 cultivation

Nodulisporium sp. KT29 cultivation was directed using Tarman *et al.* (2011) method). The method was begun with the *Nodulisporium* sp. KT29 isolate rejuvenation in potato dextrose agar (PDA) for seven days in 28–30°C. The result of preculture was used in three different treatments, *V. harveyi* dead cell induction of *Nodulisporium* sp. KT29 (NM), *V. harveyi* active cell induction of *Nodulisporium* sp. KT29 (NH), and without induction (NT). *V. harveyi* suspension was prepared in 300 mL of sea water complete (SWC) broth and incubated in waterbath shaker for 18 hours in 28–30°C with 160 rpm. The NM treatment was prepared using autoclave in 121°C for 30 minutes before mass culture.

The result of *Nodulisporium* sp. KT29 preculture was collected separately 12.5 mL (5%) and mixed with *V. harveyi* dead cell induction (NM) 12.5 mL (5%), *V. harveyi* active cell induction (NH) 12.5 mL (5%), and no cell induction treatment (NT). The mass cultured was conducted in 250 mL of PDB for 14 days using shaker with 120 rpm and 28–30°C.

The collection was done at day 15 using whatman filter paper (mesh size 0.45 μm) and then the *Nodulisporium* sp. KT29 sample was evaporated (in 40°C) to separate water content and a 50 mL from 250 mL *Nodulisporium* sp. KT29 metabolite was reserved. As many of 20 mL of the sample was used in the treatments (NT, NM, and NH).

The preparation of antibacterial testing used maceration method to obtain the *Nodulisporium* sp. KT29 metabolite. The maceration method used EtOAc (ethyl acetate) with ratio 1:2 (100 mL of *Nodulisporium* sp. KT29 : 200 mL of EtOAc) for 3×24 hours in shaking condition (120 rpm), then evaporated (in 40°C) and reserved 1 mL).

The tested feed preparation

This study used commercial feed. The basal feed (positive and negative control feed) was repelleted with 0.1% (1 g/kg) of vitamin C addition and carboxyl methyl cellulose (CMC) as binder (3%, 30 g/kg). The tested feed was also repelleted with *Nodulisporium* sp. KT29 metabolite addition based on the treatment, i.e. NT (*Nodulisporium* sp. KT29 without *V. harveyi* cell induction), NM (*Nodulisporium* sp. KT29 with 5% of 20 mL/kg of *V. harveyi* dead cell induction), and NH (*Nodulisporium* sp. KT29 with 5% of 20 mL/kg of *V. harveyi* live cell induction). After being extruded, the feed was crumbled into smaller pieces (crumbs).

Preparation and lethal concentration₅₀ (LC₅₀) of *Vibrio harveyi*

The Koch Postulate was conducted towards *Vibrio harveyi* to increase its virulence using injection method to a 5 g of Pacific white shrimp on the 3rd abdomen. *Vibrio harveyi* then reisolated by streaking the inoculation loop on the hepatopancreas and intestine of the shrimp, then streaked it to culture the bacteria on sea water complete agar and incubated for 24 hours in 28–30°C. The determination of lethal concentration (LC₅₀) was managed using serial dilution, i.e. 10⁷ CFU/mL, 10⁶ CFU/mL, 10⁵ CFU/mL, and 10⁴ CFU/mL. It was done for 7 days. The 10⁷ CFU/mL was got as the LC₅₀ for the resistance test.

Container preparation and resistance test

The experimental shrimp was reared in the offshore floating net cage. The floating net cage sized in 3×3 m². Inside the floating net cage, smaller net cage sized in 1×1×2 m³ was placed.

Post larvae 10 (average weight 0.014 ± 0.01 g and average length 0.8 ± 0.01 cm) was adapted for a day after stocked in the floating net cage (stocking density: 1000 ind/m³). The experimental shrimp was reared for 30 days. The feeding method was at satiation with feeding frequency 4 times a day (06.00, 10.00, 14.00, and 18.00)

The resistance test was conducted on PL₄₄ (average weight : 0.48 ± 0.07 g, average length : 4.82 ± 0.65 cm) with stocking density 30 ind/aquarium. It was managed in laboratory scale in the Fish Health Laboratory. The size of the aquarium was 30×20×20 cm³. The concentration of *V. harveyi* was 10⁷ CFU/mL (LC₅₀ result) with ratio 1:9 (100 mL of bacteria : 900 mL of sea water). The resistance test was conducted for 7 days. During the test, the shrimp was fed using at satiation feeding method and 4 times a day feeding frequency (07.00, 11.00, 15.00, and 19.00). The immune response and stress observation was done before resistance test (H0), 1st day (H1), 4th day (H4), and 7th (H7).

The experimental parameter

Antibacteria activity

The antibacterial testing or inhibition zone was managed to detect the ability of active compound *Nodulisporium* KT29 in inhibiting *V. harveyi*. The inhibition zone was tested using sea water complete agar containing 150 μL of *V. harveyi*. The dosage of each treatments was 20 μL .

Resistance of *Litopenaeus vannamei*

The resistance or survival of the tested *Litopenaeus vannamei* was calculated based on equation below (Daniels *et al.*, 2010). The observation of was done for seven days:

$$RS (\%) = \frac{Fn}{In} \times 100$$

Note :

RS = resistance (%)
Fn = final population
In = initial population

The immune response of *L. vannamei* towards *V. harveyi*

The observation of immune response was conducted before infected (H0), day 1 (H1), day 4 (H4), and day 7 (H7). The immune response parameters were phenoloxidase activity (PO) (Liu & Chen, 2004) and respiratory burst

(RB) (Cheng *et al.*, 2004). The phenoloxidase activity was measured through dopachrome forming data documentation which resulted by dihydroxyphenylalanine (L-DOPA). The experimental *L. vannamei* was crushed and the crustacea anticoagulant was added (1:2). The crushed shrimp was centrifuged for 10 minutes in 3,500 rpm. The natant substance was collected then rinsed using 1 mL of cacodylate-citrate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, and trisodium citrate 0.10 M, pH 7.0). Furthermore, it was centrifuged again using the identical speed and period, then the supernatant was dismissed. A 200 μ L cacodylate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, calcium chloride 0.01 M, and magnesium chloride 0.26 M, pH 7.0) was added and afterward a 100 μ L was discarded. The 100 μ L of cell suspension was incubated with 50 μ L of trypsin (1 mg/mL cacodylate buffer) as an activator for 10 minutes, then added by 50 μ L of L-DOPA (3 mg/mL of cacodylate buffer), incubated again for 5 minutes, and as many of 800 μ L of was added cacodylate buffer. As many of 200 μ L of the result was collected in a microplate reader and measured its optical density using 490 nm of wavelength.

The RB activity was measured based on the nitro blue tetrazolium (NBT) reduction as superoxide anion (O_2^-) measurement. The experimental *L. vannamei* was crushed and the crustacea anticoagulant was added (1:2). As many of 300 μ L of the substance was collected and incubated for 30 minutes in room temperature. Moreover, it was centrifuged for 20 minutes in 3,500 rpm. The natant layer was then added by a 100 μ L 0.3 % NBT (1 capsule was dissolved in 3 mL of PBS) and incubated in room temperature for 1 hour. The mixture was then centrifuged over for 10 minutes in 3,000 rpm. The supernatant layer was discarded and added 100 μ L of methanol absolute. Moreover, it was centrifuged again for 10 minutes in 3,000 rpm and the supernatant was discarded. The natant layer was rinsed twice using methanol 70%, then it was added by 120 μ L of potassium hydroxide (KOH 2 M) and 140 μ L of DMSO (dimethyl sulfoxide). The result was collected as many of 200 μ L into the microplate reader and it was measured its optical density (OD) in 630 nm of wavelength.

The stress response caused by V. harveyi

The observation of stress response was conducted before infection (H0), day 1 (H1),

and day 7 (H7) post infection of *Vibrio harveyi*. The stress response was measured through the hemolymph glucose content. The analysis of hemolymph glucose was managed using glucose liquicolor kit through CHOD-PAP (enzyme colometric test for glucose method with deproteinization) method. As many of 100 μ L of the hemolymph was collected and added 200 μ L of crustacea anticoagulant (1:2) and centrifuged for 20 minutes in 6,000 rpm. The supernatant was collected and moved to the other microtubeto be analysed. The hemolymph glucose was calculated using the following equation :

$$GH = \text{AbsSt}/\text{AbsSp} \times \text{GST}$$

Note:

GH = hemolymph glucose concentration (mg/mL)

AbsSp = sample absorbance

AbsSt = standard absorbance

GST = concentration of standard glucose (mg/dL)

Data analysis

The study used completed randomized design consisted of 5 treatments and 3 replications. The quatitative parameter (inhibition zone, immune responses (PO and RB), and stress responses) was tabulated using Ms. Excel and SPSS. A significant difference was analysed using Duncan posthoc test in confidence level of 95%. Meanwhile, clinical symptoms were analysed descriptively.

RESULTS AND DISCUSSIONS

Results

Antibacterial activity

The result of inhibition zone (in vitro) shows the *Nodulisporium* sp. KT29 was able to increase the inhibition zone reached up to 9.3–13 mm with the best treatment was NM and NH (13 ± 2.6 mm and 12 ± 1.0 mm, respectively). The inhibition zone of each treatment is shown below in Table 1.

The resistance of Litopenaeus vannamei

The result of this study presented that *Nodulisporium* sp. KT29 supplementation in the tested feed for 30 days was able to boost the resistance of the tested shrimp infected by *V. harveyi*. The most excellent result was presented by NM treatment ($72 \pm 2.5\%$) which was significantly different with the other treatments

Table 1. Antibacterial activity of *Nodulisporium* sp. KT29

Treatment	Dosage (μL)	Diameter of inhibition zone (mm)
Chloramphenicol (+)	20	9.3 ± 0.6^b
EtOAc (-)	20	0.0 ± 0.0^a
NT	20	9.3 ± 1.1^b
NM	20	13.0 ± 2.6^c
NH	20	12.0 ± 1.0^c

Note : Antibacterial activity of *Nodulisporium* sp. KT29 in dosage 20 μL . Treatments: chloramphenicol (positive control), EtOAc (negative control), NT (*Nodulisporium* sp. KT29 without *V. harveyi* induction), NM (*Nodulisporium* sp. KT29 with 5% of *V. harveyi* dead cell induction), and NH (*Nodulisporium* sp. KT29 with 5% of *V. harveyi* live cell induction). Different superscript in the same row indicates significant difference ($P < 0.05$).

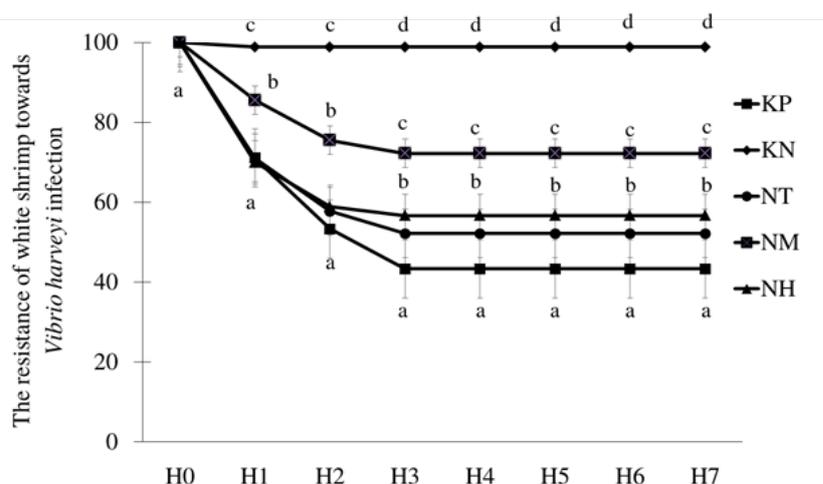


Figure 1. Mortality phase of Pacific white shrimp towards *V. harveyi* infection for 7 days. The average weight and length were 0.48 ± 0.07 g and 4.82 ± 0.65 cm, respectively. KP (positive control), KN (negative control), NT (*Nodulisporium* sp. KT29 without *V. harveyi* induction), NM (*Nodulisporium* sp. KT29 with 5% of *V. harveyi* dead cell induction), and NH (*Nodulisporium* sp. KT29 with 5% of *V. harveyi* live cell induction). Different superscript indicates significant difference ($P < 0.05$).

($P < 0.05$), except NH ($56.7 \pm 3.3\%$) and NT ($52.2 \pm 1.9\%$) treatment. According to the result, mortality happened on the first day (H1) until the fourth day (H4) post infection. The pattern of the resistance of Pacific white shrimp is shown below in Figure 1.

Phenoloxidase (PO)

The result of phenoloxidase (PO) showed that Po activity pre infection (H0) was relatively stable amongst treatments. On the first day (H1), PO activity increased, then decreased on the 4th and 7th day in the NM treatment. The PO activity is presented in Figure 2A.

Respiratory burst (RB)

Supplementation *Nodulisporium* sp. KT29 in the feed was able to rise the respiratory burst (RB). The RB activity rose on the first day (H1) post infection with the best result was shown by NM treatment ($P < 0.05$). RB activity in the NM

treatment decreased on the 4th and 7th day. The graph of RB activity was presented below in Figure 2B.

Hemolymph glucose

The hemolymph glucose before *V. harveyi* infection was considerably stable amongst treatment. On the first day, the NT, NM, and NH presented lower level of hemolymph glucose than KP treatment. The identical condition was also found on the seventh day (H7) ($P < 0.05$). The result of hemolymph glucose is described in Figure 3.

Clinical symptoms of *L. vannamei*

The clinical symptoms of *L. vannamei* caused by *V. harveyi* infection showed redness on the hepatopancreas, muscles and body was whitish. On the contrary, the healthy shrimp looked dark and the hepatopancreas was in dark color as well.

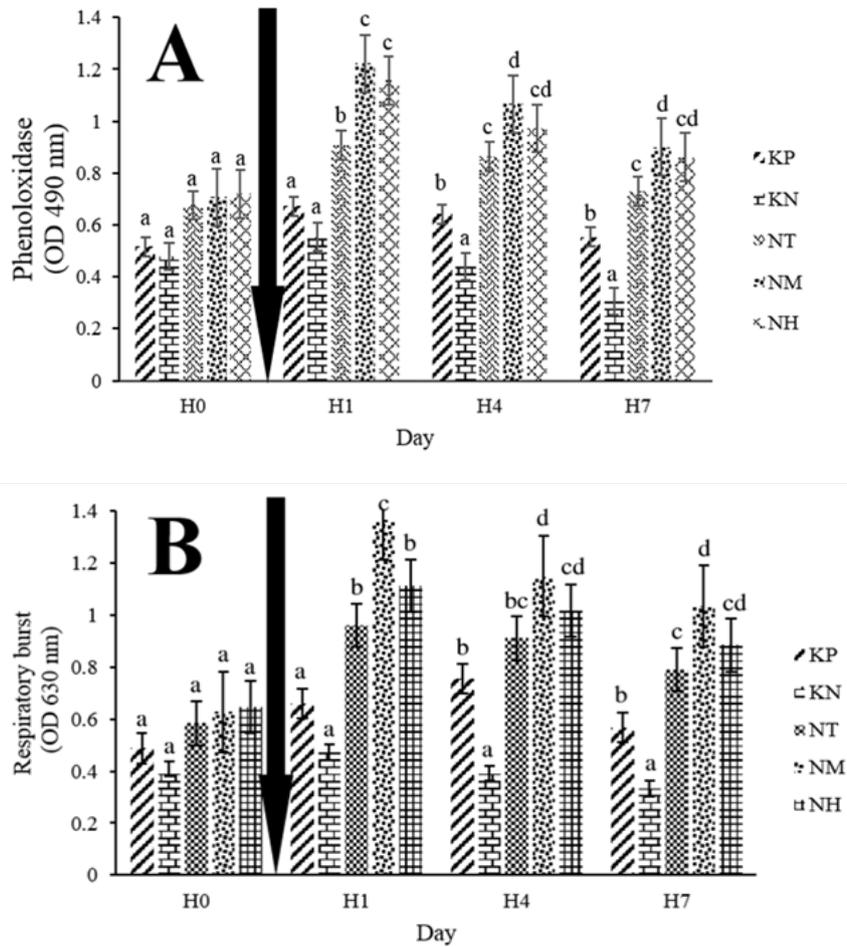


Figure 2. The activity of immune response (phenoloxidase and respiratory burst) of *L. vannamei* (average ± SE) after infected by *V. harveyi*. The observation was conducted before resistance test (H0), challenged test (↓), first day of resistance test (H1), fourth day (H4) and seventh day (H7) of resistance test. Different superscript indicates significant different (P<0.05) amongst treatment.

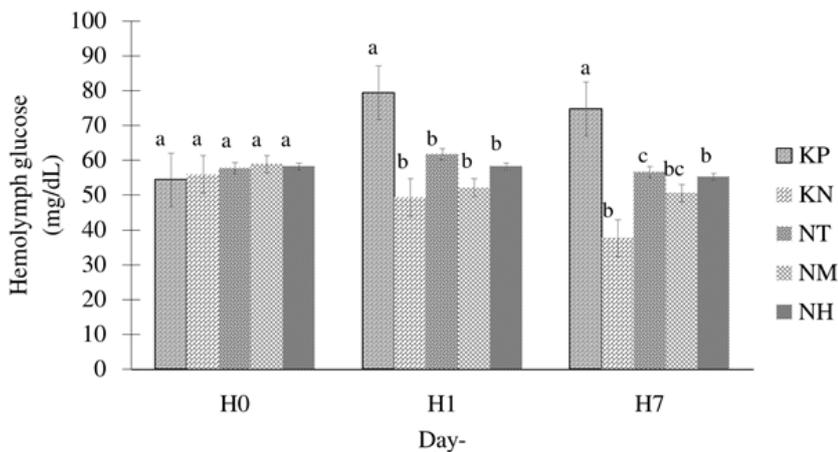


Figure 3. Hemolymph glucose of *L. vannamei* (average ± SE). The measurement was conducted before resistance test (H0), challenged test (↓), day 1 of resistance test, and day 7 of resistance test. KP (positive control), KN (negative control), NT (*Nodulisporium* sp. KT29 without *V. harveyi* induction), NM (*Nodulisporium* sp. KT29 with 5% of *V. harveyi* dead cell induction), and NH (*Nodulisporium* sp. KT29 with 5% of *V. harveyi* live cell induction). Different superscript indicates significant difference (P<0.05).

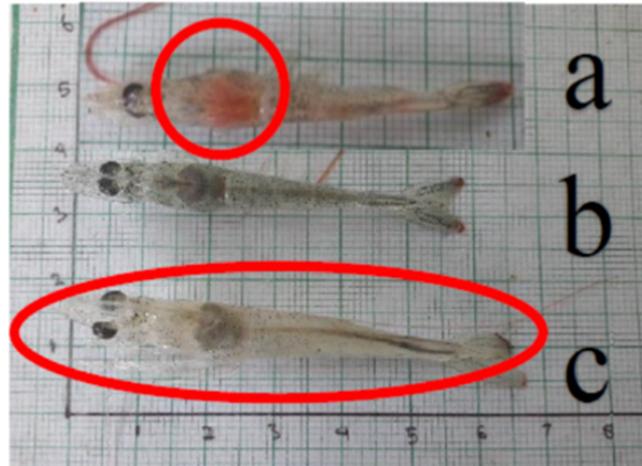


Figure 4. Clinical symptoms of *L. vannamei*. The a and c letter is infected shrimp, while b is healthy shrimp.

Discussion

The application of *Nodulisporium* sp. KT29 resulted in higher result of inhibition zone (Table 1). Bansemir *et al.* (2006) mentioned that inhibition zone is divided into 2 different kinds, low level (1–8 mm) and high level (8–15 mm). The antibacterial activity is related to saponin compound which inhibit pathogenic bacteria growth. The mechanism are antagonistic activity towards pathogenic bacteria, take over the nutrition, breakdown the cell wall, and competition of area and nutrition (Schubert *et al.*, 2008).

The active compound of *Nodulisporium* sp. KT29 which inducted *V. harveyi* is closely linked to antibacterial activity. The NM treatment was considered to have higher level of saponin, thus led to a better result in inhibition zone. The antibacterial activity holds the bactericidal mechanism which is able to destruct and kill the pathogenic bacteria. The increase of the active compound along with dead cell induction is in line with Tarman *et al.* (2011) mentioned that *Mycellium sterium* which inducted 5% of *Staphylococcus aureus* (inactive colony) increased the active compound *Nodulisporium* sp. KT29. The induction method was alienated because of the competition in nutrition and environment. The other methods to rise the secondary metabolite of fungi were mutualism, neutralism, and antagonism (Cooke & Whipps, 1993).

The administration of *Nodulisporium* sp. KT29 improved the resistance of *L. vannamei* towards *Vibrio harveyi* with the best result was NM treatment (Figure 1). The contrast in shrimp

resistance amongst treatment was caused by different active compound. It could be explained by the high level of saponin and β -glucan as antibacterial compounds in the NM treatment. Saponin played as antibacterial in the organism by attaching the cell permeability membrane towards pathogenic bacteria (Couto *et al.*, 2014). Meanwhile, β -glucan is a polysaccharides bond through β - (1,3) and glucoside β - (1,6) which commonly found in the bacteria and fungi cell wall (Bai *et al.*, 2010). The β -glucan plays role in enhancing *Penaeus lasticulatus* (Hai & Fotedar, 2009), *Cherax tenuimanus* (Sang & Fotedar, 2010), and *Litopenaeus vannamei* (Bai *et al.* 2010; Chen *et al.*, 2016) growth and immune system.

Morphological changes in the Figure 4 consisted of redness on the hepatopancreas, muscles and body became whiter, anorexia, and slow movement. Direct physical contact between shrimp and the *V. harveyi* causes a direct access for the *V. harveyi* to the digestive tract of the shrimp which causes redness on the hepatopancreas (Yeh & Chen, 2009; Widanarni *et al.*, 2012). On the other hand, a healthy shrimp has darker color, active movement, and higher appatite. It indicated that *Nodulisporium* sp. KT29 supplementation boosted the immune system using active compound, such as saponin and β -glucan. The β -glucan is able to induce nonspecific immune system by enhancing lysozyme activity in both fish and shrimp (Paulsen *et al.*, 2001; Saputra *et al.*, 2016).

Litopenaeus vannamei is categorized as crustacean which has adaptive non specific immune system and innate immune response towards bacteria (Sang *et al.*, 2011). The non-

specific immune system is able to induct bacteria membrane cell, β -glucan, lypopolisaccharides, and peptidoglicant. About the pattern recognition protei, β -1.3-glucan binds protein (BGBP), LPS and protein bind β -1.3-glucan (LGBP). This activity activates serine proteases, which is proPO activating factor (PPA), then it is converted proPO into PO (phenoloxydase) through phagocytosis activity (Li & Xiang 2013; Amparyup *et al.*, 2013; Chaosomboon *et al.*, 2016).

Phenoloxydase (PO) plays role as humoral immune response in crustacean and it is also known as non-specific extracellular immune system mechanism to prevent any infection caused by pathogenic bacteria. Hemocyte takes part in PO mechanism by blocking harmful compound getting into the shrimp (Li & Xiang, 2013; Amparyup *et al.*, 2013). The PO activity in the NM activity showed higher increase compared to the other treatments (Figure 3A). The result showed that β -glucan takes essential part in PO activity in inducing the immune system by granular cell to capture unusual compound which invades the shrimp. The mechanism consist of proPO transformation into PO and inactive serine protease (proppa) into serine protease (ppa) which is activated by β -glucan and peptidoglicant to catalize monophenol and phenol oxidation in producing quinones. The quinones act as encapsulation and melanisation towards unfamiliar compounds (Li & Xiang, 2013; Amparyup *et al.*, 2013). The PO activity is also known as unknown substances detector in humoral non-specific immune system (Li & Xiang, 2013; Hauton, 2012). The increase of PO activity demonstrated that immunostimulant administration was able to induce the resistance and immune system of *Litopenaeus vannamei* towards *V. harveyi* infection (Zubaidah *et al.*, 2015; Wahjunigrum *et al.*, 2016).

Respiratory burst is a cellular immune response and intracellular defense in the non-specific immune system with phagocytosis activity towards unfamiliar compound (Mai & Wang, 2010; Hauton, 2012). The RB in the NM treatment described higher increase compared to the other treatment (Figure 2B). The result indicated that β -glucan as the active compound was able to boost the phagocytosis in inducing immune system by hyaline phagocyte cell in the phatogen debris to produce reactive oxygen mechanism (ROI). The phagocytosis activity

involves the superoxide anion (dependent oxygen mechanisms) degradative enzyme releasing and the production of ROI which also known as respiratory burst (Mai & Wang, 2010; Hauton, 2012). The mechanism of phagocytosis activity are attachment (direct contact) and ingestion of unfamiliar compound that will form digestive vacuole called phagosome. Phagosome and lysosome (hyaline phagocytes) merge and form phagolysosome which later will be destructed by unfamiliar object in the cell through ingestion process (dependent oxygen mechanism) and produce ROI. The ROI activity takes part in coordinating cortisol hormone towards glucose activity caused by the biological factor as the environment adjustment indicator (Chen *et al.*, 2015).

The increase level of RB was considered as beneficial result. It is related to the shrimp immunity towards pathogen infection. However, an excessive increase level of RB is regarded as an unfortunate condition for the host (Mai & Wang, 2010). The other studies also reported the beneficial factor of the immunostimulant to several different species, such as *Cyprinus carpio* (Lin *et al.*, 2012), common carp (Djauhari *et al.*, 2016), and *Pangasianodon hypophthalmus* (Tamamusturi *et al.*, 2016). Meanwhile, the decrease of immune response indicated recovery cell against *V. harveyi*. The later statement is supported by Huang *et al.* (2013) who stated that a low level of immune response is a recovery indicator of the organism.

Hemolymph glucose (GH) is a secondary stress indicator towards physiological activity and immune system indicator against the phatogen infection on shrimp and fish (Bulfon *et al.*, 2013). The NM treatment (20 mL) was capable to minimize glucose content compared with the positive control (KP) on the post infection (Figure 3). The polyphenol active compound which stored in *Nodulisporium* sp. KT29 carries out the decreasing of hemolymph glucose post-infection of *V. harveyi*. Polyphenol acts as antioxidant defense of the shrimp and cell protection against free-radical caused by oxidative stress (Wang *et al.*, 2015). *Crustacean hyperglycemic hormone* (CHH) is responsible for hemolymph activity in crustaceans and physiological behaviour (Chen *et al.*, 2014). Stress condition triggers the hypothalamus to secrete CHH which will lead to inhibit insulin production (Chung *et al.*, 2010). Glucose mechanism occurs through

glycogenolysis. Glicogenolysis is biochemical breakdown of glucogen to glucose during stress condition and low level of insulin in neutralizing glucose content as stress response (Zhou *et al.*, 2011).

CONCLUSION

Vibriosis control in *L. vannamei* using *Nodulisporium* sp. KT29 supplementation is considerably potential to increase the resistance and immune system. The most stand out result is presented by the NM treatment (induction of *Vibrio harveyi* dead cell and dosage 20 mL/kg feed).

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