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Immune response and growth performance of crayfish *Cherax quadricarinatus* fed with synbiotic supplemented diet

Performa respons imun dan pertumbuhan lobster air tawar *Cherax quadricarinatus* yang diberi pakan mengandung sinbiotik

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ABSTRACT

This study aimed to investigate the effectiveness of synbiotic (prebiotic alginate and probiotic bacteria *Micrococcus* spp) on the immune responses, growth performance, and bacterial resistance of freshwater crayfish. The experimental diets were formulated in three levels of synbiotic: the probiotic bacteria *Micrococcus* spp+100 mg/L alginate (Syn100), *Micrococcus* spp+200 mg/L alginate (Syn200), *Micrococcus* spp+300 mg/L alginate (Syn300), each level was mixed with tested feed. The basal diet (without synbiotics) served as the control diet. During 40 days of rearing, immune responses observations were conducted every 10 days, while the crayfish weight was recorded on day 40. Furthermore, the challenge test was performed against the pathogenic bacteria of *Aeromonas hydrophila* and reared for a week. The result showed that synbiotics treatment of Syn300 and Syn200 could improve the immune response and increased the growth rate (P<0.05). Both were also higher than that of the Syn100 treatment, while the lowest was the control. The highest resistance to *A. hydrophila* pathogenic bacteria by demonstrating a higher survival rate was Syn300 and followed by Syn200 (70.00 ± 0% and 73.33 ± 5.77%, respectively), compared to Syn100 (56.67 ± 3.33%) and the control (33.33 ± 3.33%). The synbiotic *Micrococcus* spp. combined with alginate potentially induced immune responses, increased growth performance, and improved bacterial pathogens resistance, making it an ideal synbiotic to be developed.

Keywords: synbiotic, prebiotic, probiotic, *Sargassum* sp., alginate, brown algae

ABSTRAK

Penelitian ini bertujuan untuk menguji efektivitas penggunaan sinbiotik (prebiotik alginat dan probiotik *Micrococcus* spp) terhadap respons imun, performa pertumbuhan dan resistansi lobster air tawar terhadap bakteri patogen. Tiga perlakuan sinbiotik, yaitu: *Micrococcus* spp+100 mg/L alginat (Syn100), *Micrococcus* spp+200 mg/L alginat (Syn200), *Micrococcus* spp+300 mg/L alginat (Syn300), masing-masing dicampurkan ke dalam pakan dan diberikan ke lobster. Pakan tanpa campuran prebiotik digunakan sebagai pakan kontrol. Selama 40 hari pemeliharaan, pengamatan respons imun dilakukan setiap 10 hari, dan pada hari ke-40 pemeliharaan dilakukan pengukuran bobot lobster. Selanjutnya, lobster diuji tantang dengan bakteri *A. hydrophila* dan dipelihara kembali selama satu minggu. Hasil penelitian menunjukkan bahwa pemberian pakan yang mengandung sinbiotik Syn300 dan Syn200 dapat meningkatkan respons imun dan pertumbuhan (P<0,05) lebih tinggi dibandingkan dengan perlakuan Syn100, sementara bobot tubuh terendah didapat pada perlakuan kontrol. Resistensi lobster tertinggi terhadap bakteri *A. hydrophila* yang ditunjukkan dengan sintasan tertinggi didapatkan pada perlakuan Syn300 (70,00 ± 0,00%) dan Syn200 (73,33 ± 5,77%) dibandingkan dengan perlakuan Syn100 (56,67 ± 3,33%) dan kontrol (33,33 ± 3,33%). Penelitian ini membuktikan bahwa sinbiotik *Micrococcus* spp. yang dikombinasikan dengan alginat dapat menginduksi respons imun, meningkatkan performa pertumbuhan, dan resistansi terhadap patogen sehingga merupakan sinbiotik yang ideal untuk dikembangkan.

Kata kunci: sinbiotik, prebiotik, probiotik, *Sargassum* sp., alginat, alga coklat

INTRODUCTION

The disease is a major threat to aquaculture production. Nonetheless, it mostly deals with antibiotic for controlling disease outbreak. It leads to resistance of pathogenic bacteria in various species and potentially leads to the environmental destruction. Therefore, preventive action through improvement of the fish or prawn's immunity is highly crucial. Various studies related to the application of vaccines have been documented, including whole cell vaccines and extracellular products (Pasnik *et al.,* 2005), extracellular proteins (Song *et al.,* 2013), and the 89 kDa protein extracellular vaccine (Amrullah *et al.,* 2014a; 2014b). Studies about immunostimulants, among them, referring to LPS characterization (Cai *et al.*, 2013), flagellin from *Marinobacter algicola* and *Vibrio vulnificus* (Montero *et al.,* 2014), and nutritional impact on fish mucosa (Caipang & Lazado, 2015) have been reported.

In addition, vaccines, immunostimulants, and probiotics have played various important roles in aquaculture and resulted numerous developments (Sharifuzzaman *et al.,* 2014; Park *et al.,* 2016; Speranza *et al.,* 2017), leading to the development of "prebiotics" such as mannan oligosaccharides (MOS), fructooligosaccharides (FOS), inulin and vitamin C (Torrecillas *et al.*, 2007; Zhou *et al.,* 2010; Ibrahem *et al.,* 2010; Munir *et al.,* 2016), probiotics (*Bacillus coagulans)* and prebiotics B-GOS galactooligosaccharides) and fat intake in modulating the immune response (Liu *et al.,* 2017). The most current developments are nutritional supplements, combinations of a probiotic and a prebiotic which are called synbiotics. Synbiotics have demonstrated more effective and long-lasting effects on growth and immunological responses (Van Doan *et al.,* 2016; Wang *et al.,* 2017; Safari & Paolucci, 2017; Hindu *et al.,* 2018). Similarly, Sewaka *et al*. (2019) found that the synbiotic application on the juvenile of red tilapia (*Oreochromis* spp) caused the intestines villus becoming high and wide. This leads to the expansion of food absorption area and feeds efficiency.

The brown algae (*Phaeophyceae*) contain polysaccharides such as laminaran, fucoidan, and alginate (Blanco-Pascual *et al.,* 2014) in addition to mannitol and traces of cellulose (Chang *et al.,* 2010; Seon *et al.,* 2014). Alginate is a complex combination of oligo-polymer, mainly consisting of polymannuronic and polyglucuronic acids (Blanco-Pascual *et al.,* 2014). Alginate is found in the cell wall where the main supportive material located, comprising up to 40% of the algae's dry weight (Draget *et al.,* 2005; Jung *et al.,* 2013; Seon *et al*., 2014). The application of alginate from brown algae in human increase gastroprotective and reduce the lesions occurrence in the human stomach (Ammar *et al*., 2018; Qin, 2018) and as prebiotics, antimicrobials, immunomodulation (Dierick *et al.,* 2010). Applications on tilapia (Van Doan *et al.,* 2017) and sea cucumbers (Wang *et al.,* 2017) using alginate from brown algae have been performed and showed positive response in growth performance and immune system.

The freshwater crayfish, *Cherax quadricarinatus*, is a type of crayfish which commonly cultivated in Indonesia with a lot of economic potentials. However, its slow growth rate and susceptibility to bacterial diseases, especially *Aeromonas hydrophila*, are crucial issues in intensive lobster cultivation systems. In addition, a high density and limited area which cause stress in the animals, encourage the development of diseases and causing slow growth and might end in mass mortality. In order to overcome those issues, synbiotics is a plausible alternative way required for biological control (Wang *et al*., 2017).

The bacteria *Micrococcus* spp. (coded BTL) that isolated from the intestines of *C. quadricarinatus* grow well in media containing alginate in vitro (Amrullah *et al.,* 2014c); therefore, the present study aimed to investigate the effectiveness of the synbiotic using the combination of the *Micrococcus* spp as potential probiotics and the prebiotic alginate extracted from brown algae *Sargassum crassifolium.* In addition, some parameters measured such as growth performance, immune response, and resistance of freshwater crayfish to pathogenic bacteria. Furthermore, the health status and disease of *C. quadricarinatus* were also observed using immune response and A. hydrophila resistance towards synbiotic supplementation.

MATERIALS AND METHODS

Experimental crayfish

The freshwater crayfish *C. quadricarinatus,* in similar age groups, were obtained from a number of crayfish cultivation centers in South Sulawesi, Indonesia. The average body weight of the crayfish used for the experiment was 9.45 ± 47 g.

Prebiotics bacteria and prebiotic preparation

Prebiotics was extracted from alginate of brown algae *S. crassifolium*. Probiotic bacteria

Micrococcus spp. were isolated from the intestines of freshwater crayfish *C. quadricarinatus*, the best probiotic bacteria chosen based on in vitro testing (Amrullah *et al.,* 2014c). The challenge test was done with a virulent strain of *Aeromonas hydrophila* bacteria. The crayfish was acclimatized under aerated conditions for a period of 14 days at 28°C. The lobsters were fed a commercial lobster feed using *ad libitum* feeding method twice a day during the acclimatization period.

Alginate extraction

The alginate extraction of *S. crassifolium* was performed according to Murdinah *et al.* (2005). Dried *S. crassifolium* was immersed in 1% HCl 1:30 b/v for 1 hour. Chemical extraction was conducted with 2% Na_2CO_3 (1:30) at 60–70°C for 60 minutes. The sample was grounded and re-extracted at 60–70ºC for 60 min. Next, the extracts were blended with NaOCl (4% of filtrate volume) for 30 min. Alginic acid was obtained with the addition of 10% HCl at pH between 2.8 and 3.2. In addition, alginate acid was washed to neutral. A 10% NaOH was used for Na-alginate conversion with pH 7–8 and continued with Na-alginate separation in IPA (1:2 v/v), stirred for 30 minutes. Moreover, oven drying at 50°C for 12 hours was applied. The sample was grounded to powder to obtain alginate flour.

Experimental diet preparation

The basal diet contained 30% protein, 5% fat, 4% fiber, and 12% moisture. Three different experimental feeds were used by adding alginate 100, 200, and 300 mg/L for each. One control feed group was provided for the experimental animal (without any prebiotic). The resulting dough was cold-extruded through a palletiser with an appropriate size. The pellets were air-dried and stored at room temperature in air-tight containers. The feed was then sprayed with *Micrococcus* spp. The experimental feed was stored in sealed plastic bags at 4°C before used for the further experiment.

Experimental design and culture system

A completely randomized design was applied for a laboratory experiment. Three different experimental feeds were used; probiotic bacteria *Micrococcus* spp + 100 mg/L alginate (Syn100), *Micrococcus* spp + 200 mg/L alginate (Syn200), and *Micrococcus* spp + 300 mg/L alginate (Syn300) which had three replication for each. While four different rearing periods of synbiotic treatment were used, 10 days, 20 days, 30 days, and 40 days. The basal diet (without synbiotics) was served as the control diet. After acclimation, the experimental animals were reared in fiber glass tanks (40 L capacity) at a density of 15 crayfish per aquarium. Feeding was conducted twice a day using ad libitum method. The crayfish were reared for 40 days and observations of immune responses were made every 10 days and they were weighed on day 40.

Challenge test

The freshwater crayfish were reared in fiber glass tanks (40 L capacity) with a density of 10 freshwater crayfish/tank triplicate and fed twice a day at a feeding rate of 5% of body weight. After 40 days of the feeding trial, the crayfish were challenged with 10^6 CFU/mL the bacteria *A. hydrophila*. The survival rate of the lobsters was observed on the 7th day post-challenge test.

Statistical analysis

Data survival rate, body weight, total hemocyte count, differential hemocyte count, and phagocytic activity were statistically analyzed using one-way ANOVA with SPSS 26.0 (SPSS Inc. IL) program. Significant different ($P<0.05$) was further tested with the Duncan test.

RESULTS AND DISCUSSION

Results

Total hemocyte count

The prebiotics alginate mixed with the probiotic bacteria *Micrococcus* spp as a synbiotic (Figure 1), could increase the total hemocyte count which was demonstrated by the distribution of total hemocyte in each treatment which increased until day 40. The average total hemocyte count in treatment was between 8.5–3.9 ($\times 10^6$ cells/mL), while control treatment was 3.17–2.97$\times 10^6$ cells/mL).

The statistical analysis results showed there was a significant difference in level and treatment duration of symbiotic on total hemocyte count. Nonetheless, there was no interaction between the synbiotic concentration and treatment duration ($P<0.05$). The main effect of synbiotics concentration on average total hemocyte response for 40 days regardless of the effect of treatment duration indicated that the treatment of 200 mg/L (5.94) and 300 mg/L (5.78) were significantly different ($P<0.05$) and higher than the the treatment of 100 mg/L (5.01) and control (3.08). Furthermore, the main effect

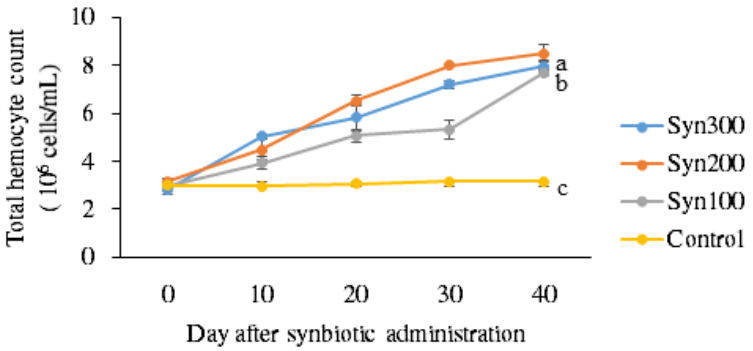

Figure 1. The average of total hemocyte count of crayfish Cherax quadricarinatus after being fed synbiotic-enriched feed with different level of synbiotic and duration of treatment (mean±SD). Different superscript letters indicate significant differences among treatments (P<0.05).

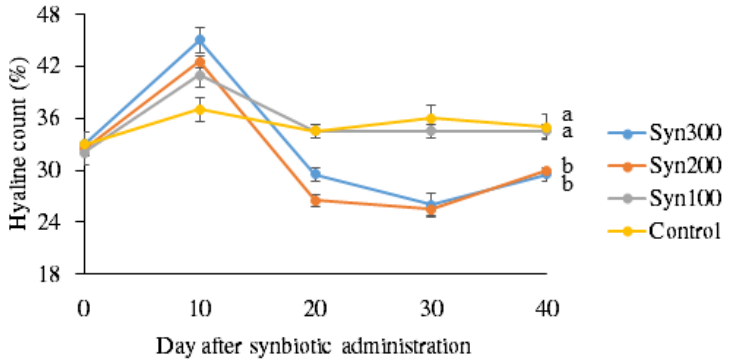

Figure 2. Average hyaline count of crayfish Cherax quadricarinatus after being fed synbiotic-enriched feed with different level of synbiotic and duration of treatment (mean±SD). Different superscript letter indicate significant differences between treatments (P<0.05).

Table 1. Average semi granulocyte count of crayfish *Cerax quadricarinatus* after being fed synbiotic-enriched feed with different level of synbiotic and duration of treatment.

Different level of synbiotic (mg/L)	Duration of treatment (day)	Average semi granulocyte count of crayfish *Cerax quadricarinatus* (%)			
		I	II	III	IV
Syn200	day-10	40.00			
Syn300	day-10	39.50	39.50		
Syn300	day-0	37.00	37.00		
Syn200	day-0	36.50	36.50		
Syn100	day-0	36.50	36.50		
Control	day-0	36.50	36.50		
Control	day-40	35.50	35.50		
Syn100	day-10		34.50		
Control	day-10		34.50		
Syn100	day-20		34.00	34.00	
Syn100	day-40		34.00	34.00	
Syn300	day-30		33.00	33.00	
Control	day-30		33.00	33.00	
Control	day-20		32.00	32.00	
Syn100	day-30		31.00	31.00	
Syn200	day-20		30.00	30.00	
Syn200	day-30			29.00	29.00
Syn300	day-40			27.00	27.00
Syn300	day-20			24.50	24.50
Syn200	day-40				22.50

Different column indicate significant differences among treatments (P<0.05)

Table 2. Average granulocyte count of crayfish *Cherax quadricarinatus* after being fed synbiotic-enriched feed with different level of synbiotic and duration of treatment.

Different level of synbiotic (mg/L)	Duration of treatment (day)	Average granulocyte count of crayfish *Cherax quadricarinatus* (%)		
		I	II	III
Syn200	day-40	47.50		
Syn300	day-20	46.00		
Control	day-30	45.50		
Syn300	day-40	43.50		
Syn200	day-20	43.50		
Syn100	day-30	41.00		
Control	day-20		33.50	
Syn100	day-0		31.50	
Syn100	day-20		31.50	
Syn100	day-40		31.50	
Syn300	day-30		31.00	
Syn200	day-0		31.00	
Control	day-0		30.50	
Control	day-40		30.50	
Syn300	day-0		29.50	
Syn200	day-30		29.50	
Control	day-10		28.50	
Syn100	day-10		27.50	
Syn200	day-10			18.50
Syn300	day-10			15.50

Different colom indicate significant differences among treatments (P<0.05).

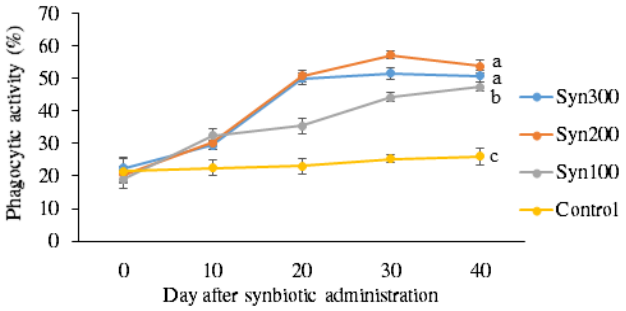

Figure 3. Average phagocytic activity of crayfish *Cherax quadricarinatus* after being fed synbiotic-enriched feed with different level of synbiotic and duration of treatment (mean±SD). Different superscript letters indicate significant differences among treatments (P<0.05).

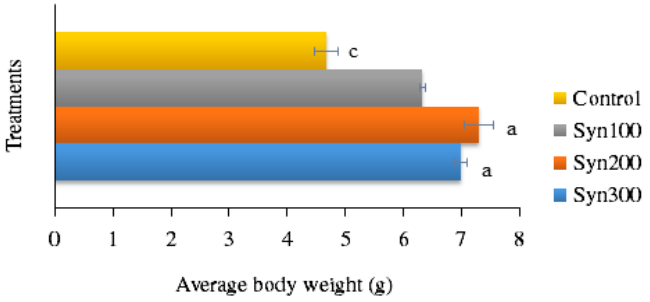

Figure 4. Average body weight of crayfish *Cherax quadricarinatus* after being fed synbiotic-enriched feed with different level of synbiotic and time duration (mean±SD). Different superscript letters indicate significant differences among treatments (P<0.05).

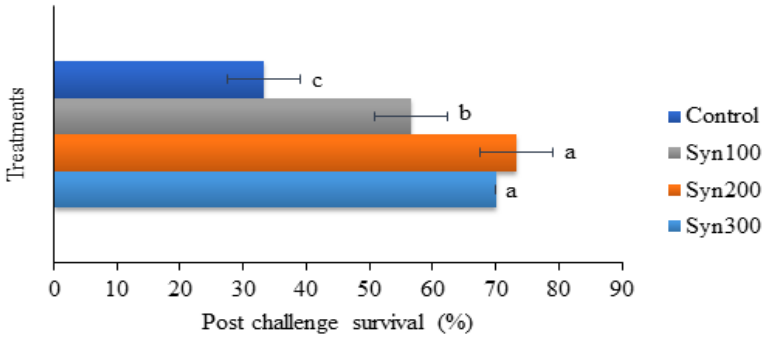

Figure 5. Average percent survival of crayfish *Cherax quadricarinatus* fed synbiotic-enriched feed (the probiotic Micrococcus spp. and the prebiotic alginate) for 40 days after the challenge test with the bacteria Aeromonas hydrophila (mean±SD). Different letters indicate significant differences among treatments (P<0.05).

of the treatment duration showed that the more synbiotics gave, the higher total hemocyte of the crayfish which was observed at day 40. It showed higher the total hemocytes and significantly different from the treatment duration (Figure 1).

Differential hemocyte count

It was discovered that the mean percentage of hyaline, semi-granular, and granular of crayfish *C. quadricarinatus* were change respectively after synbiotic administration of various level and duration of symbiotic, as illustrated in Figures 2, Table 1 and 2. The percentage of hyaline and semi-granular of crayfish went down and otherwise, an increased in granular cells after application of synbiotic. The decrease of hyaline and semi-granular cells was only in the form of non-numbered percentages, which seen with an increase in total hemolymph. The decline of hyaline and semi-granular cells was due to a large increase in the percentage of granular crayfish cells.

The mean percentage of hyaline for 40 days fed with Syn100 (34.7%) and control (35.1%) showed greater increased (P<0.05) than those of the other treatments. The main effect of the treatment duration of synbiotic on crayfish showed that treatment duration of 10 days (40.63%) was better than those reared for 40 days (32.25%), 20 days (31.25%) and 30 days (30.50%). No interaction between synbiotics concentration and duration of treatment.

Statistically, the semi granulocyte cell parameter showed that there was a difference between level of synbiotic and treatment time. In addition, there was an interaction between the level of synbiotic and the length of administration (P<0.05). The main effects of different synbiotics on the semi-granulocyte response, in general, the treatment of Syn200 and Syn300 were higher than the Syn100 and control on day 10. Meanwhile, the administration of Syn100 and control for 20–40 days increased significantly the semi-granular of crayfish.

In the granulocyte parameter, the synbiotic application was also different based on synbiotic concentration and duration of administration (P<0.05). Both treatments showed interaction with granulocyte crayfish (P<0.05). The highest granulocytes (P<0.05) occurred on day 20–40 with Syn200 and Syn300 applications.

Phagocytic activity

The mean phagocytic activity for the different level of synbiotic and time duration of crayfish is shown in Figure 3. The crayfish phagocytic activity began to increase on day 10 and was highest (P<0.05) between day 30 and 40. In general, the average hemocyte activity of crayfish for 40 days in crayfish fed synbiotic-enriched feed Syn300 and Syn200 (42.5% and 40.85%, respectively) was higher than that of crayfish which did not feed with synbiotics (P<0.05). The duration of 30 days (44.56%) and 40 days (40.85%) of the synbiotics administration increased phagocytic activity compared to 20 days (39.81%) and 10 days (28.68%) of treatment. There was no interaction between the level of the synbiotics and the treatment duration on phagocytic activity.

Body weight

The administration of synbiotics leads to a higher body weight of crayfish (Fig. 4) than in crayfish fed synbiotic-standard diet (P<0.05). After being fed synbiotic-enriched feed for 40 days, the body weight of the crayfish fed the synbiotics Syn300 and Syn200 (6.99±0.11 g; 7.30±0.25 g, respectively) were heavier than the crayfish fed the synbiotics Syn100 (6.33 ± 0.04 g), and the lowest body weight was control (4.67 ± 0.20 g) (P<0.05).

Survival rate

The survival rate of the crayfish reared for 40 days by experimental feed and then tested for the challenge with *A. hydrophila* can be seen in Figure 5. The administration of synbiotics in the treatment of Syn300 and Syn200 indicated mortality (respectively with SR 70.00±0.00%; 73.33±5.77%) which was significantly higher ($P<0.05$) compared to Syn100 (56.67±3.33%) of crayfish, and the lowest was obtained in control (33.33±3.33%).

Discussions

The results of this study demonstrated that the synbiotics applied through feed could induce nonspecific immune responses and increase body weight of crayfish. Furthermore, due to the crayfish' immune responses increased after the administration of the synbiotics, they could protect the crayfish from *A. hydrophila* infection in the challenge test. This was proven by the higher survival rate in the crayfish fed synbiotics after the challenge test compared to the crayfish without any synbiotics. This present finding also suggests that probiotic and prebiotic are synergistic combination obtained from one of supplement nutrient. Thus, this synergistic combination can be alternative preventive measures and control of bacterial disease in fish farming (Sewaka *et al*., 2019).

The total hemocyte in crayfish fed synbiotic-enriched feed containing the prebiotic alginate was higher than the control (Fig.1). This was also seen in the percentage of granulocytes and hemocyte activity which increased and were higher in crayfish fed synbiotic-enriched feed ($P<0.05$) compared to those not fed synbiotics. In general, all the immunological experiment parameters increased significantly after the administration of synbiotic-enriched feed. The opposite was observed in the negative control which was fed synbiotic-free feed. The results demonstrated that probiotics presented in the form of synbiotics could increase immune responses, especially non-specific responses (Azimirad *et al.,* 2016; Wang *et al.,* 2017). Administration of the synbiotic for 40 days could increase the crayfish' body weight more than the control. The probiotic bacteria balanced the micro-flora in the lobsters' digestive tract, improved the absorption of nutrients and as a result improved the nutritional value of the feed. This phenomenon was not seen in crayfish not fed synbiotics. This study is in line with Sewaka *et al.* (2019) who studied the JA + LGG synbiotic supplementation in tilapia. Due to the development of the intestinal villi is better than the treatment without synbiotics, synbiotics can expand the nutrient absorption areas and more effective. The number of microvilli widens the absorption area and the food can be circulated by the blood throughout the body. Therefore, good quality food is able to meet the energy requirement for maintenance and energy reserves for growth.

Probiotic bacteria hold an important role in the digestive tract. These bacteria thrived in the digestive tract and produce inhibitor compounds which could suppress the growth of harmful bacteria (Fjellheim *et al.,* 2007) and produce digestive enzymes which could aid the digestion process (Gatesoupe, 1999; Shang *et al.,* 2018). Therefore, the proteins and energy from the feed could be utilized more efficiently by the crayfish. As a result, the administration of synbiotics leads to better growth performance. In less than ideal nutritional conditions, but having a high probiotic bacteria population in the digestive tract, the probiotic bacteria could improve the host's survival rate because they are able to increase feed digestibility and provide digestive enzymes or vitamins (Dillon & Dillon, 2004; Ringø & Song, 2016; Elshopakey *et al.,* 2018).

The probiotic bacteria which found in the gut effectively maximized its own energy harvested from these glycans (Flint *et al.,* 2008). This process formed an integrated metabolic network in the gut, and eventually, SCFAs and secondary metabolites that produced during the fermentation process were beneficial to the intestinal ecology and host physiology. In addition to the use of probiotics, the role of prebiotics also begins to be studied. For instance, the role prebiotic in fructooligosaccharide on post-larvae giant freshwater prawn *Macrobrachium rosenbergii* (Chen *et al.,* 2017). Li *et al.* (2018) reported the use of inulin and MOS combination in white shrimp, and Elshopakey *et al*. (2018) use β-1,4-mannobiose on kuruma shrimp (*Marsupenaeus japonicus*). All the results showed positive effects on growth and immune response. However, most of the tested prebiotic is a commercial product, whereas the natural raw material from marine is not often found (Wang *et al.,* 2016).

Alginate is a major structural polysaccharide of brown algae, which has physicochemical properties beneficial to intestinal ecology (Holdt & Kraan, 2011). The polysaccharide generates a beneficial bioactive compound with a variety of chemical structure based on alga species

(Wijesekara e*t al.*, 2011). Furthermore, it is easily fermented by certain bacteria in the gut. This fermentation resulted in short-chain fatty acids acts (SCFA) as an important source of energy for epithelial and immune cells.

Alginate contains high nutritional value, various bioactive compounds, and minerals (Cabrita *et al.*, 2016). As prebiotic, alginate is an indigestible food ingredient which beneficial for parasite organism activity, leading to increasing growth and nonspecific immune responses (Poongodi *et al.*, 2012; El-Desouky *et al.*, 2012; Peso-Echarri *et al.*, 2012; Schleder *et al.*, 2017), increasing lipid metabolism (Güroy *et al.*, 2011), viruses resistance and resistance (Boonsri *et al.*, 2017), increasing intestinal effectiveness (Michiels *et al.*, 2012), and increasing the resistance of organisms to stress (Nath *et a.l*, 2012). In addition, alginate also potentially increases the abundance of *bacteriides capillosus* bacteria in the gut (Kuda *et al*., 2017) and has antiviral, antifungal and antibacterial activity (Niu *et al.*, 2015).

Study on several marine algae species have been conducted in aquaculture including *Undaria pinnatifida* (Schleder *et al.*, 2017), *Sargassum philippendula* (Pallaoro *et al.*, 2016), *Ulva lactuca* and *Gracilaria parvispora* (Rodríquez-González *et al.*, 2014), *Hypnea cervicornis,* and *Cryptoremia crenulata* (Silva & Barbosa, 2009). The results of those studies are in line with the utilization of natural ingredients and natural antibiotic which is environmentally friendly. Various studies have also been documented related to the use of prebiotic and probiotic bacteria. It showed good results on growth and immune response. However, if probiotic and prebiotic bacteria are applied together in the form of synbiotics, it will produce advanced results compared to the provision of prebiotics as a single compound (Safari *et al.,* 2017).

The application of synbiotic with different level of prebiotic resulted in dissimilar growth performance and immune responses in the lobsters. The application of the prebiotics at concentrations of 200 and 300 mg/L (Syn300 and Syn200) in general could improve the crayfish' growth performance, immune responses, and survival rate compared to those lower concentration of prebiotics (100 mg/L or Syn100). These results demonstrated that 200 mg/L and 300 mg/L of alginate were the optimum concentration to boost growth performance, immune responses, and survival rate in crayfish, whereas administration of 100 mg/L synbiotic was inadequate. According to the treatment duration of synbiotics, it could be given during the rearing periods. This was based on data from the synbiotics administration which showed a higher difference on day 30 and 40 than other time treatments on all test parameter.

The survival rate of lobsters after 40 days of being fed using synbiotic-enriched feed then challenged with a virulent strain of *A. hydrophila* was higher than that of the crayfish fed the non-synbiotic-enriched feed. The results of this study demonstrated that the probiotic bacteria *Micrococcus* spp. could indeed induce the lobsters' immune response. Sufficient amounts of the prebiotics fucoidan and alginate supported the existence of probiotic bacteria in the crayfish's digestion tract, which in turn induced immune responses strong enough to protect the crayfish against infections of *A. hydrophila*.

The total hemocyte, an immune response parameter, could affect the crayfish's ability to react to unfamiliar matter and various responses to infection (Johansson *et al.,* 2000); therefore, the increased total hemocyte resulted in improved health in the lobster. Hemocytes play a role in the process of phagocytosis, encapsulation, degradation, and nodular aggregation of pathogens and foreign bodies. This total hemocyte indicated the ability of the crayfish to defend itself against pathogens. In crustacean, the Increase of hemocyte level indicates resistance in the body which attempts to remove the pathogens that infected the body through phagocytosis. In the present study, synbiotic alginate and Micrococcus spp can stimulate an increase in THC level. The optimal THC level can be gained at 200 and 300 mg/L. This study is in line with Partida-aragure *et al.* (2013) with the administration of inulin in white shrimp.

In the present study, the differential hemocyte parameters increased. The increased differential hemocyte is an indication of the crayfish's elevated health status or improved resistance. Hyaline cells which have an active role in phagocytosis and encapsulation and proPO, cytotoxic, granulocytes, which accumulated in connective tissue, were easily released into the hemolymph to perform proPO and cytotoxic functions (Johansson & Soderhall, 1989). The increase of granulocyte crayfish cells after the application of alginate prebiotics and probiotic bacteria *Micrococcus* sp. had an effect on the increase of phagocytic activity. Granulocytes are

important phagocytic cells in crayfish in order to conduct phagocytosis of foreign particles. After being challenged with *A. hydrophila*, the crayfish fed synbiotic-enriched feed had significantly higher survival rates (P<0.05). This demonstrated the fact that non-specific immune responses performed defence mechanisms which were activated by the probiotic bacteria, making the crayfish more resistant to pathogenic bacteria.

The use of seaweed as a beneficial nutrient has been widely developed for human needs because it acts as an antimicrobial compound (Pina-Perez *et al.,* 2017), cancer prevention, blood pressure reduction, and antioxidants (Qin, 2018). Furthermore, the use of alginate from seaweed as synbiotic on tilapia (*O. niloticus*) has been studied by Van Doan *et al.* (2017) using alginate and probiotic bacteria *Lactobacillus plantarum*. The results showed that this synbiotic potentially increased innate immune response, disease resistance, and growth performance. The application of alginate as prebiotic has also been studied by Kuda *et al.* (2015) against *Salmonella typhimurium*, *Listeria monocytogenes,* and *Vibrio parahaemolyticus* infections in mice. This alginate administration increases transepithelial resistance from HT-29-Luc monolayer cells and decreases the number of pathogens in the liver.

A study on the effects of the administration of probiotics and commercial prebiotic on growth has been documented by Talpur *et al.* (2014). The results of the study demonstrated that the administration of *L. acidophilus*, yeast, and ß-glucan could significantly increase body weight, protein efficiency ratio (PER), and feed conversion ratio (FCR) in fish. The administration of the prebiotic fructooligosaccharide (FOS) could also improve immune responses, stress resistance, digestive activities, and growth in the larvae of Caspian roach fish (Soleimani *et al.,* 2012). The probiotics in synbiotics increase the secretion of alginate lyase enzymes, amylase, and proteases (Zokaeifar *et al*., 2012). Synbiotic can also regulate the balance of intestinal microbes lead to accelerating digestion and absorption of feed (Gomez & Balcazar, 2008).

This is also in line with Shang *et al.* (2018) who stated that intestinal microbiota plays an important role in health, nutrition, metabolic, and immune homeostasis of the host. As symbiotic bacteria, these microorganisms strongly rely on fiber and polysaccharides that cannot be digested as an energy source. Polysaccharides reach the gut are fermented by intestinal microbiota lead to a fundamental impact on the intestinal ecology. Therefore, alginates from *Sargassum* sp. brown algae and *Micrococcus* spp bacteria are important as synbiotics for fish cultivation, especially freshwater crayfish.

CONCLUSION

The synbiotics potentially induced immune responses, improve body weight, and the resistance to the pathogenic bacteria *A. hydrophila* in crayfish; the best concentration for the prebiotic was at 200 mg/L and for the probiotic bacteria *Micrococcus* spp. at a density of 10^5 cells/mL.

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