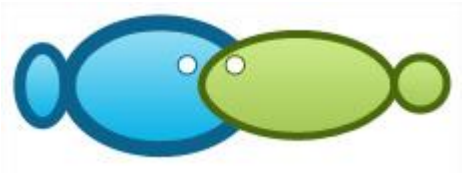

Local synbiotic from *Amorphophallus muelleri* Bl. and *Bacillus* sp. to boost *Litopenaeus vannamei* non-specific immune responses

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Abstract. Disease management is still an obstacle in *Litopennaeus vannamei* culture. For example, in 2009, a new disease appeared in brackish water shrimp species, namely EMS (Early Mortality Syndrome) caused by *Vibrio parahaemolyticus* with a unique strain (Vp-AHPND) plasmid (pAP1) 70 kbp. The syndrome could decrease the survival rate of *L. vannamei* dramatically after infection. This study aimed to examine the effect of synbiotic (*Amorphophallus muelleri* Bl. and *Bacillus* sp.) mixed in the commercial feed to boost *L. vannamei* immune system against *V. parahaemolyticus*. The study was conducted with 4 treatments, including T0 (commercial antibiotic), T1 (3% prebiotics), T2 (2% probiotics and 3% prebiotics), T3 (4% probiotic and 3% prebiotic), T4 (6% probiotic and 3% prebiotic). Furthermore, all data was analyzed using statistical analysis that is provided by SPPS program. The results showed that the administration of synbiotics consisting of 2% probiotic and 3% prebiotic increased most parameters, including total hemocyte count (THC), granulocytes cells, total weight, and survival rate reaching 13.4 x 10^6 cells mL^{-1}, 58.67 %, 0.182 g, and 50 %. Moreover, the highest number of phagocytosis was generated by T1, accounting for 65.67 %, while four treatments (T0, T1, T3, and T4) had a similar level for hyaline. Based on the results, the present study suggests that 2% probiotic and 3% prebiotic (T2) could be applied in the *L. vannamei* culture as a synbiotic to improve the immune system and growth performance.

Key Words: combination, growth performance, immune system, survival rate.

Introduction. Vannamei shrimp (*Litopenaeus vannamei*) is one of Indonesia's fishery commodities and an export commodity (Amelia et al 2021). Based on statistical data from the General Directorate of Aquaculture Indonesia, the production of vanamei shrimp reached 517.397 tons in 2020. Furthermore, the production of vannamei shrimp is projected to climb, achieving 1.290.000 tons in 2024 (a rise of 250%) (Soebjakto 2020). However, shrimp production increases with intensive shrimp cultivation always negatively impact the environment and generate some diseases, such as bacteria, viruses, fungi, and protozoans (Gao et al 2016; Pang et al 2019; Putra & Romdhonah 2019). In 2009, in China, a new disease appeared in brackish water shrimp species, namely EMS (Early Mortality Syndrome) caused by the bacterium *Vibrio parahaemolyticus* with a unique strain (Vp-AHPND) plasmid (pAP1) 70 kbp (Lestiawan et al 2016). According to Soto-Rodriguez Sonia et al (2015), genus *Vibrio* sp. causes vibriosis generating necrosis to shrimp tissues, particularly the hepatopancreas. Moreover, *V. parahaemolyticus* could produce a toxin that leads to Acute Hepatopancreatic Necrosis Disease (AHPDN), which causes the shrimp death within 20-30 days (Khimmakthong & Sukkarun 2017; Kumar et al 2020).

Therefore, it is necessary to find a solution for ensuring the success of shrimp production through increasing shrimp immunity (Munaeni et al 2020). Commonly, shrimp farmers use chemicals or antibiotics to combat pathogens and boost immunity, leading to an environmental hazard and increasing resistance to bacteria (Cheng et al 2020; Munguia & Nizet 2017; Van Hai 2015). Recently, many researchers have been concerned

about using medicinal plants or probiotics to promote the shrimp immune system (Kewcharoen & Srisapoome 2019; Ringø & Song 2016; Zhang et al 2020). For instance, *Bacillus aryabhattai* supplemented in commercial feed could enhance immune parameters, including phenoloxidase (PO) activities and the total antioxidant activity in the *L. vannamei* plasma. Moreover, *B. aryabhattai* also upregulated some immune-related genes, such as C-type lec, pen3a, hsp60, trx, and fer (Tepaamorndech et al 2019). Total hemocyte count, PO activity, phagocytic activity, and O_2^- production of *L. vannamei* also enhanced after feeding trial with 20 g of *Phyllanthus amarus* extract (PAE) to prevent *Vibrio alginolyticus* (Ngo et al 2020). In other exciting work, bacteria (10^8 cfu *Bacillus* sp.) and IMOS, isomalto-oligosaccharides (2 g kg^{-1}) both in one combination improved various vital body processes such as enhanced hemocyte phagocytosis, respiratory burst abilities, phenoloxidase, acid phosphatase, alkaline phosphatase production, and disease resistance to WSSW (Ringø & Song 2016). The combination of dietary synbiotic was studied by Kumar et al (2018), and *Bacillus subtilis* and the prebiotic Mannan oligosaccharide (MOS) promoted *Cirrhinus mrigala* juveniles physiologically and immunologically resistance to *Aeromonas hydrophila*. In addition, there was a combination between 5 g kg^{-1} Jerusalem artichoke (*Helianthus tuberosus*) combined with 10^8 CFU g^{-1} *Lactobacillus plantarum* boosted specific growth rate, feed conversion ratio, serum lysozyme, phagocytosis, respiratory burst activities, and post-challenge survival rate compared with the control group (Van Doan et al 2016). Therefore, the present study aimed to evaluate a combination between *Bacillus* sp. and *Amorphophallus muelleri* Bl. to *L. vannamei* immunity and its resistance to *V. parahaemolyticus*. *Bacillus* sp. have been studied as a probiotic bacteria for any purpose, such as boosting shrimp immunity and increasing feed digestibility and utilization (De et al 2018; Laranja et al 2017; Madani et al 2018). However, there is still a limitation of *A. muelleri,* related to promoting shrimp immunity and its function as a prebiotic. Yanuriati et al (2017) says *A. muelleri,* could be determined as having a high potential of glucomannan, which plays a critical role in food, pharmaceutical, cosmetics and chemical industries. Glucomannan hydrolysate could be a beneficial prebiotic that modulates the development of probiotic bacteria, promotes the formation of short-chain fatty acids, and optimizes gastrointestinal health (SCFAs) of the host (Li et al 2021).

Material and Method

Experimental design. From June to August of 2021, the study was carried out at the Fisheries Laboratory of the University of Muhammadiyah Malang in Indonesia. Furthermore, the experiment employed a completely randomized design (CRD) with four treatments replicated three times. Those treatments included (T0) 6 mL of synthetic antibiotic (followed factory instruction), (T1) the addition of 0% probiotics and 3% prebiotics, (T2) addition of 2% probiotic and 3% prebiotic, (T3) addition of 4% probiotic and 3% prebiotic, (T4) addition of 6% probiotic and 3% prebiotic per 1 kg of commercial feed. Furthermore, the feeding trial was carried out for 14 days to promote *L. vannamei* immunity before the shrimp sample was challenged with *A. hydrophila*. At the end of the period, the present study evaluated some immune systems, such as total hemocyte count (THC), differential hemocyte count (DHC), and phagocytosis index (IF). At the same time, growth rate (GR) and survival rate (SR) were determined to support measurements.

Prebiotic, and probiotic preparation. The preparation of liquid media from *A. muelleri* Bl. referred to Azhari et al (2021) study. In brief, *A. muelleri* Bl. tubers were peeled and washed before being sliced and soaked in water. Following the step, the sliced *A. muelleri* Bl was introduced in calcium carbonate ($CaCO_3$) with a ratio of 1:1. Afterwards, *A. muelleri* was sliced again in 1 mm to 2 mm slices, to speed up the drying process. Furthermore, the thin *A. muelleri* Bl. were dried in the oven at 50°C to get a constant weight. Finally, the dried sample was grounded and filtered with 100 to 120 (0.125 millimeter to 0.149 millimeters) mesh to yield *A. muelleri* Bl. flour.

Moreover, the preparation of *A. muelleri* Bl. liquid medium was carried out by inserting 0.5 g of *A. muelleri* Bl. flour into a beaker glass containing 50 mL of distilled water, according to Praseto (2015). After that, the mixture was homogenized using a magnetic stirrer before being heated on a hot plate. The boiled mixture was poured into a 200 mL Erlenmeyer and sterilized in an autoclave for 25 min at 121ºC. The sterile liquid media was stored at room temperature.

This study used *Bacillus* sp. as a probiotic agent, synergized with *A. muelleri* Bl. liquid medium, and a commercial probiotic available in the society. The probiotic bacteria (10^8 CFU mL^{-1}) was provided by the Parasite and Fish Disease Laboratory, Faculty of Fisheries and Marine Science, Brawijaya University, Malang, East Java, Indonesia. Meanwhile, the commercial antibiotic (Inroflox 25) was bought from the local fisheries market, Malang, East Java, Indonesia.

Experimental shrimp, and diets preparation. All preparation in this section referred to Wijayanti et al (2018) and Ramadhani (2017) methods with light modifications. In the first step, the aquarium (40 cm×30 cm×30 cm) was disinfected using 30 ppm chlorine for 24 h before being filled with ¾ sterile seawater of the total volume. Meanwhile, the experimental *L. vannamei* (12 g to 15 g) were provided from shrimp ponds in the Pasuruan, East Java, Indonesia. A total of 10 *L. vannamei* were acclimated in a container according to each treatment and were fed with a commercial diet (28 % to 38 % protein) ammounting to 3 % of the total body weight for 3 days.

The feed treatment was prepared by making a combination 1 kg of commercial diet and each treatment, completed with 2% binder agent. Following the next step, the feed treatments were air-dried for 10 min–15 min under sunlight to reduce water content and increase binding among particles. Afterwards, the diet was stored in an airtight container. The present study conducted four feeding trials with as much as 3% of the total shrimp biomass in each treatment.

Challenge and survival rate evaluation. The Parasite and Fish Disease Laboratory, Faculty of Fisheries and Marine Science, Brawijaya University laboratory, provided *V. parahaemolyticus* (10^8 CFU ml^{-1}) used in this study. The challenge test was conducted after the 14 days feeding trial, and the survival rate was recorded for each 7 days. Survival rate (SR) is the percentage of shrimps still alive after the challenge test. The survival rate of shrimp can be calculated using the following formula from the research of Nur et al (2016):

$$SR = \frac{N0 - Nt}{N0} x100$$

where:
SR = Survival rate of shrimp (%)
Nt = number of dead shrimps after the challenge test
No = number of shrimps at the beginning of the study

Hemolymph collection. Hemolymph was carefully taken from the cephalothorax between the walking and swimming legs (the beginning of the 5th pereiopod) as much as 0.3 mL using a sterile syringe (1 mL) containing anticoagulant (10 % Sodium Citrate). Afterwards, the collected hemolymph was homogenized and placed in 2.0 mL Eppendorf™ tubes. The mixture was then stored in a cool box to observe the shrimp immune response parameters (Anaya-Rosas et al 2019; Subagiyo & Fatichah 2016). The sampling was carried out on three shrimp at the end of the period.

Non-specific immune response. Hemolymph (10 µL) was diluted with PBS (20 µL) gently. After that, the mixture was transferred to a hemocytometer using a micropipette. Finally, the number of THC was calculated based on Permatasari (2017) formula:

$$\text{THC (cells } mL^{-1}) = \text{number of cells counted x dilution x } 10^4$$

The hemolymph and anticoagulant mixture was homogenized for 5 min and then dripped into a glass object. The diluted hemolymph was air-dried and then fixed with 100% methanol for 15 min. Following the next step, the fixed hemolymph was air-dried and stained with 10% Giemsa solution for 15 min. The stained hemolymph was washed in running water for 30 s and allowed to dry again. The slides were observed using a microscope with a 40× magnification to record the number of hyaline and granular cells. The formula calculated the percentage of each type of hemocyte cell from Indraswati et al (2015):

$$\text{Percentage of hemocyte cell types} = \frac{\Sigma\, Type\ hemocyte}{Hemocyte\ total} \times 100\%$$

Moreover, the phagocytosis index followed a method from Ramadhani et al (2017) and Permatasari (2017). In the first step, the fresh hemolymph (20 µL) was put in a microtube before adding a 10 µL suspension of *Staphylococcus aureus*, attenuated with 1% formalin for 24 h. Afterward, the mixture was incubated at room temperature for 20 min, and then a 5 µL sample was smeared on the object-glass. The dried slide was soaked in 70% alcohol for 20 min and rinsed 0.85% NaCl then dried again.

Furthermore, the slide was painted with 10% safranin for 20 min and dried. The observation was conducted under a microscope with a magnification of 400x. Phagocytic activity (PA) was calculated using Permatasari (2017) formula:

$$PA = \frac{\Sigma\, Phagocyte\ cell}{\Sigma\, Hemocyte\ total} \times 100\%$$

Absolute weight. The growth of the final weight of the tested shrimp can be calculated using the formula according to Edward et al (2015), namely:

$$Wm = Wt - W0$$

Wm = Absolute weight (g)
Wt = Average weight of shrimp at the end of the study (g)
W0 = Average weight of shrimp at the beginning of the study (g)

Statistical analysis. Analyzing differences among groups was performed using ANOVA (One-way analysis of variance). Multiple comparisons (BNT test) were carried out to determine significant differences among treatments using SPSS (version 17, USA). Data were displayed as the mean ±SD, $p<0.05$ was considered significant.

Results

Total hemocyte count (THC). In the case of *L. vannamei* THC, it could be found that there was a significant difference ($p<0.05$) among treatments at 21 days (Figure 1). The data are represented in 10^6 cells mL^{-1}. Overall, T2 was the optimum treatment of all treatments, while the remaining treatments were not significantly different from the control group ($p>0.05$). In the top-level, T2 achieved the highest peak of THC number, reaching 13.4 × 10^6 cells mL^{-1}, after 21 days of treatment. Meanwhile, four treatments belonged to the lowest level of THC, including T0, T1, T3, and T4, accounting for 5.5 × 10^6 cells mL^{-1}, 5.6 × 10^6 cells mL^{-1}, 6.3 × 10^6 cells mL^{-1}, respectively. Based on findings, it revealed that the synbiotic had a better outcome in THC than control group, applying antibiotic.

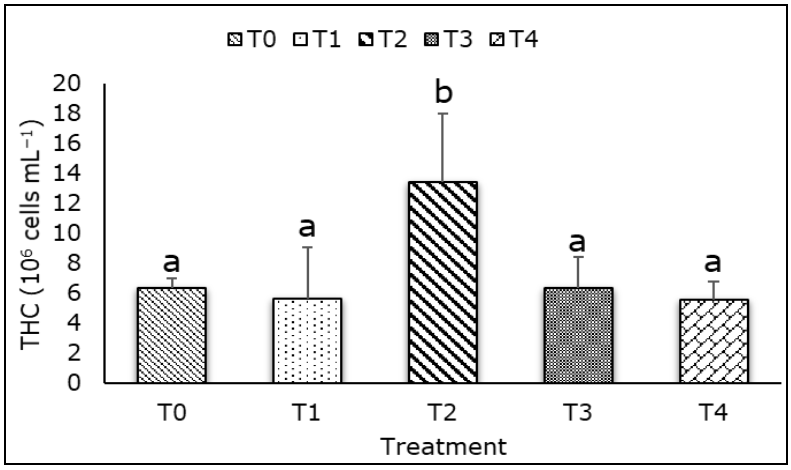

Figure 1. Total hemocytes count of *L. vannamei* post-challenge *V. parahaemolyticus*.

Differential hemocyte count (DHC). This study discovered two types of hemocytes that could be identified at the end of the period. Figure 2 reveals that the feed treatment could stimulate hyaline and granulocytes for 21 days of research. The data of this parameter are presented percentually (%). Overall, only T2 became the most recommendable treatment to incline *L. vannamei* granulocytes after post-challenge *V. parahaemolyticus*. However, T2 went to the lowest level for hyaline quantities if it was compared to others. Moreover, for another finding, the higher dosage of the synbiotics did not improve the number of granulocytes. On the other hand, in the hyaline case, the moderate dosage decreased the number of hyaline.

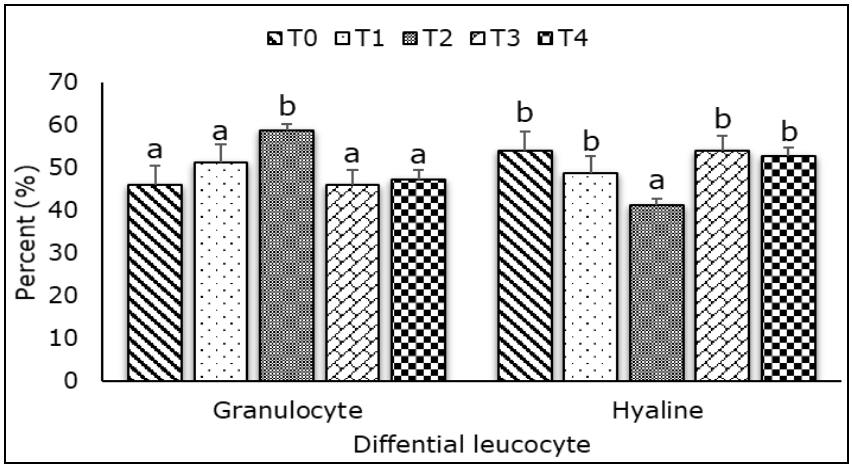

Figure 2. The number of granulocyte and hyaline of *L. vannamei* after *V. parahaemolyticus* infection.

The present study revealed that T2 could significantly stimulate granulocyte levels, reaching 58.67±1.53% after infection. Meanwhile, the remaining treatment, T1, T3, and T4, accounting for 51.33±4.16%, 46.00±3.61%, and 47.33±2.08%, did not show differences to the control group ($p>0.05$). This means that the T1, T3, and T4 did not positively affect *L. vannamei* granulocytes during infection by *V. parahaemolyticus*. Moreover, there was a considerable decline of hyaline by T2 of 41±1.5%, while T0, T1, T3, and T4 (54.00±1.5%, 48.67±1.5%, 54.00±1.5%, and 52.67±1.5%) were not that different statistically. Regarding those findings, the synbiotics (*A. muelleri* and *Bacillus* sp.) are considerable and could be introduced in the *L. vannamei* culture to improve their immune system better than antibiotic.

Phagocytosis index (IF). Analysis of phagocytosis index (IF) reveals a fluctuation pattern for 21 days observation after synbiotic treatment and challenge with *V. parahaemolyticus* (Figure 3). All IF data are presented in percentages (%). Statistically,

the IF data showed significant differences in each treatment ($p<0.05$). Overall, T1 was determined the optimum treatment to promote IF against *V. parahaemolyticus* infection. Meanwhile, the worse treatment was T3 consisting of 4% probiotic and 3% prebiotic.

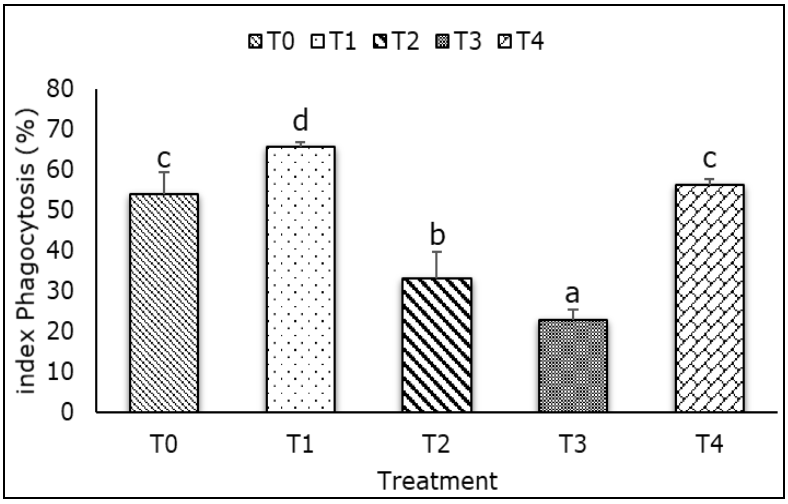

Figure 3. Phagocytosis for *L. vannamei* after 21 days observation.

The present study discovered that T1 could reach the highest peak of *L. vannamei* IF, accounting for 65.67±1.15%. Meanwhile, the second place belonged to T0 and T4 (54.00±5.41% and 56.38±1.32%, respectively) based on the statistic calculation. Furthermore, T2 and T3 became worse than other treatments, reaching 33.23±6.40% and 22.83±2.75%, respectively. According to those findings, the application of synbiotic *A. muelleri* and *Bacillus* sp. could stimulate the number of phagocyte cells, although the higher dosage did not cause a positive outcome.

Absolute weight. The present study found that the final weight of *L. vannamei* fluctuated at 21 days after treatment with synbiotic through the spray method and *V. parahaemolyticus* challenge (Figure 4). Based on this data, the treatment was ineffective to incline *L. vannamei* growth during *V. parahaemolyticus* infection, represented in absolute weight data. In general, all data did not show a significant difference compared to the control group (commercial antibiotic) ($p>0.05$). Interestingly, only P1 and P2 revealed a significantly different, reaching 0.18 g and 0.11 g, respectively. Regarding those discoveries, combination of *A. muelleri* Bl. and *Bacillus* sp. did not promote *L. vannamei* development during *V. parahaemolyticus* challenge.

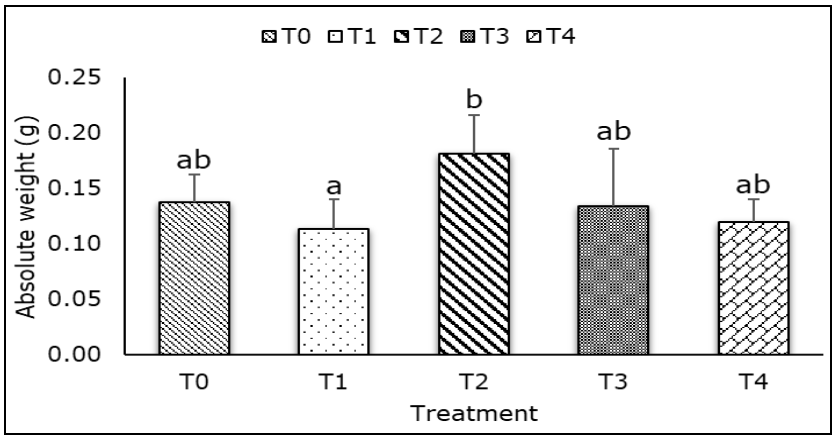

Figure 4. The absolute weight of *L. vannamei* during 21 days of observation.

Survival rate. Figure 5 shows the *L. vannamei* survival rate data at 21 days after being injected with *V. parahaemolyticus*. The data reveals significant differences among

treatments ($p<0.05$) after employing a statistical tool. All data are interpreted in terms of percentages (%). In general, T2 was the best treatment, while the least recommended was T1 to prevent *V. parahaemolyticus* infection. Moreover, survival rate data revealed that T2 could increase SR by 50%, 4% higher than the control treatment.

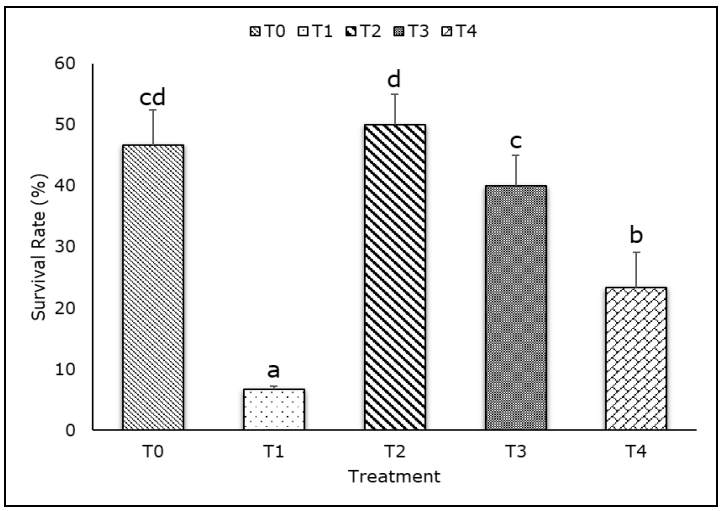

Figure 5. The survival rate of *L. vannamei* during maintenance.

Interestingly, T0 and T2 were not statistically significantly different, even though there was a gap between those two treatments, accounting for 46.67±5.77% and 50±5%. On the other hand, T0 was also not significantly different from T3, with an SR value of 40%. At the lowest level, T4 and T1 have 23.3% and 6.6%, respectively. Regarding those findings, the synbiotic treatment could keep the survival rate of *L. vannamei* after *V. parahaemolyticus* infection.

Discussion. Many studies have looked at the influence of prebiotics and probiotics on the immune system and have shown their value (Chen et al 2020; Kumar et al 2018; Lee et al 2018; Nayak 2010; Wongsasak et al 2015). Aquaculture has effectively utilized oligosaccharides and *Bacillus* as prebiotics and probiotics. Glucomannan, a type of oligosaccharides, could protect against infections, modulate immunity, and influence metabolism, as well as increasing mineral absorption (Li et al 2021; Sanders et al 2019). In this case, the *Amorphophallus* genus has potential as a probiotic because they are 49% to 60 % rich in glucomannan.

Moreover, *A. muelleri* has the potential to be proven to facilitate bacterial populations, including lactic bacteria *and E. coli,* to grow well (Harmayani et al 2014). Based on a previous study, certain bacteria of the genus *Bacillus* were good probiotics. A survey by Tepaamorndech et al (2019) discovered that oral commercial feed completed with *B. aryabhattai* TBRC8450 ($1x10^8$ CFU/g diet) treatment increased *L. vannamei* immunity against *Vibrio* spp. Therefore, finding out how prebiotics and probiotics interact with each other in aquaculture is critical. The present study has proven the success of the addition of synbiotic in natural immunity against the *V. parahaemolyticus* infection. Synbiotic supplements are a mixture of probiotics and prebiotics, which work together to level a beneficial effect on each other (Cerezuela et al 2011; Kumar et al 2018). The host determines a probiotic's synergistic effect, and the prebiotic must notably boost the probiotic's growth and enzyme activity (Huynh et al 2018).

In the case of shrimp immunity, our study discovered that the combination between *A. muelleri* and *Bacillus* sp. promoted the number of *L. vannamei* hemocytes to prevent *V. parahaemolyticus* (Figure 1). Based on Guzman et al (2009) review, dietary immunostimulants stimulate hematopoietic tissue and elevate hemocyte cells. The role of hemocytes is known for fighting disease through their protection capabilities (Jiravanichpaisal et al 2006). The presence of more THC in crustaceans indicates that their immunity has improved (Jiravanichpaisal et al 2007). Hemocytes are involved in phagocytosis, coagulation, encapsulation, nodulation, antimicrobial peptide synthesis,

and prophenoloxidase activity (Koiwai et al 2018; Ng et al 2015; Sui et al 2016). In a study by Madani et al (2018), dietary probiotic *Bacillus* enhanced the total of hemocytes count of *L. vannamei* significantly after feeding trials (60 days). *Litopenaeus vannamei* given 25% Fermented Soybean Meal with *Bacillus* sp. had a substantially greater THC ($p<0.05$) compared to all other groups (Cherdkeattipol et al 2021). In contrast, dietary supplementation with β-glucan and synbiotics (i.e., microencapsulated *B. subtilis* and *Pediococcus acidilactici*) did not positively affect THC *L. vannamei* (Wongsasak et al 2015).

Furthermore, our results revealed that granular cells (Figure 2) increased in synbiotic-fed shrimp (T2), while hyaline cells declined at the end of the period presented by T2 as well. The hyaline (HC) decrease was assumed because the compensation of granulocytes (GC) climbed considerably post-infection. It could be related to Xu Z. et al (2019) research, a sharp rise in *Penaeus vannamei* HC occurred after 9 hours of the low temperature and air exposure to compensate for a drop of GC Granulocytes (GC), whose primary function is phagocytosis, and are tasked with storing prophenoloxidase (Zhu et al 2018). In the same case, the role of Hyaline cells (HCs) is phagocytosis, a procedure that helps to remove bacteria (Chen et al 2015). Based on this study's findings, it is concluded that the synbiotic-supplemented shrimp diet improved immune function, as demonstrated by their higher number of hemocyte cells and GC cells.

In addition, the synbiotic (T1) treatment could stimulate phagocyte cells significantly than other treatments ($p<0.05$). According to Xu L. et al (2019), the increase of phagocyte cells is connected to *L. vannamei* hemocytes. Unfortunately, T1 could not increase the number of hemocytes considerably compared to the control group (T0). In contrast, immunostimulants work by enhancing the activity of phagocyte cells, which helps the body's immune system function better (Yin et al 2006). The results of increased phagocytic activity indicate that probiotic bacteria can activate phagocyte cells and are thus capable of carrying out the phagocytosis process if an attack occurs (Widanarni et al 2016). Regarding those results, the present study assumes that the addition of synbiotic consisting of *A. muelleri* and *Bacillus* sp. promoted promptly the number of phagocytic cells.

Finally, in the case of the growth performances and survival rate, T2 became the most effective treatment among other treatments for boosting those parameters at the end of the period. Discoveries supported by Liong (2008), show that probiotics and prebiotics work together to promote the host's overall health by modulating gut microbiota. In a study by Abdollahi-Arpanahi et al (2018), *B. subtilis* and *B. licheniformis* significantly influenced *L. vannamei* growth performances. In the other case, the increase of *L. vannamei* growth also found *B. subtilis* stimulated that through accelerating feed efficiency, protein efficiency ratio, and apparent net protein utilization (Tsai et al 2019). According to Leonel Ochoa-Solano and Olmos-Soto (2006), *Bacillus*'s probiotic strains can break down several substances (including proteins, lipids, and carbohydrates) via extracellular enzymes.

Moreover, regarding survival rate, it is believed that *Bacillus* sp could develop well on the *A. muelleri* media as a prebiotic and diminish *V. parahaemolyticus* by producing antibacterial substances. According to Sharma et al (2013), *Bacillus* is the most prolific antibiotic producer, producing antibacterial, antifungal, and other bioactive chemicals. This bacterium had a similar material to a bacteriocin. It demonstrated solid antibacterial capabilities in retarding Gram-positive and Gram-negative bacteria growth, including important pathogens such as *Aeromonas hydrophila* and *Streptococcus agalactiae* (Meidong et al 2018). Based on those discoveries, the combination between *A. muelleri* and *Bacillus* sp has a potential impact when applied in the *L. vannamei* farm.

Conclusions. The present study found that *A. muelleri* and *Bacillus* sp. could elevate *L. vannamei* immunity (THC, DHC, and phagocytosis index), final weight, and survival rate after *V. parahaemolyticus* infection. Overall, the T2 is determined as an optimum combination (2% probiotics and 3% prebiotics) compared to other treatments. Therefore, this study highly suggests that this synbiotic could be applied to real aquaculture to combat infectious diseases, mainly bacteria, and replace antibiotic use.

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Conflict of Interest. The authors declare no conflict of interest.

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