

RESEARCH ARTICLE

# Synbiotic containing *Kappaphycus alvarezii*, *Spirulina* sp. and *Halomonas alkaliphila* improves survival, growth and vibriosis resistance in whiteleg shrimp (*Litopenaeus vannamei*) post-larval culture

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**Abstract** – The high losses in shrimp production due to mortality caused by *Vibrio* opportunistic pathogens still constitute a significant challenge in the shrimp industry. Synbiotic feed supplementation appears to be a promising control strategy to maintain healthy shrimp stock. In this study, the effects of synbiotic-containing prebiotic seaweeds *Kappaphycus alvarezii* and *Spirulina* sp. as well as probiotic *Halomonas alkaliphila* were evaluated on the survival, growth, and vibriosis of *Litopenaeus vannamei* during the post-larval stage. Five different feeds were tested: commercial feed, prebiotics *K. alvarezii* and *Spirulina* sp.-supplemented feed, and synbiotic-supplemented feed using *K. alvarezii*, *Spirulina* sp. and probiotic *H. alkaliphila* with different concentrations of  $10^8$ ,  $10^9$ , and  $10^{10}$  CFU.kg<sup>-1</sup>. Following 14 days after the feeding test, the highest shrimp survival ( $91.46 \pm 0.05\%$ ) was obtained in the treatment group fed with synbiotic-supplemented feed containing 0.375% *K. alvarezii*, 0.125% *Spirulina* sp., and *H. alkaliphila* at  $10^9$  CFU.kg<sup>-1</sup> ( $p < 0.05$ ). A 7-day challenge test against opportunistic bacteria *Vibrio harveyi* was then performed using three treatment groups: (1) synbiotic, containing 0.375% *K. alvarezii*, 0.125% *Spirulina* sp., and *H. alkaliphila* at  $10^9$  CFU.kg<sup>-1</sup>; (2) prebiotic, with 0.375% *K. alvarezii*, 0.125% *Spirulina* sp.; and (3) control, using commercial feed. The highest shrimp survival of ( $79.9 \pm 0.05\%$ ) was found in the synbiotic treatment group, followed by the prebiotic and control treatment groups ( $p < 0.05$ ). Overall results suggested that synbiotic-supplemented feed containing 0.375% *K. alvarezii*, 0.125% *Spirulina* sp., and *H. alkaliphila* at  $10^9$  CFU.kg<sup>-1</sup> significantly improved shrimp survival even when challenged with *V. harveyi*. Thus, this synbiotic can be potentially applied as an alternative biocontrol strategy against vibriosis in intensive shrimp post-larval culture.

**Keywords:** *Halomonas* / *Kappaphycus* / Shrimp / *Spirulina* / Synbiotic / *Vibrio*

## 1 Introduction

With an average growth of 8.6% per year, aquaculture shows promising results in meeting the global demand in fish products, which is expected to increase about 50% by 2050 (Boyd and Nevin, 2015; FAO, 2018). The whiteleg shrimp *Litopenaeus vannamei* is one of the main species used in

aquaculture, particularly in South-East Asia, which makes up 65% of the global shrimp production (FAO, 2019). However, high losses in shrimp production due to mortality caused by viruses and pathogenic bacteria are still a significant challenge to meeting global production quotas (Chiu et al., 2007). One of the common diseases found in shrimp is vibriosis, a disease caused by bacteria of the *Vibrio* genus, such as *Vibrio harveyi*. This disease is known to cause 70–90% of shrimp mortality during the post-larval stage, when its concentration in shrimp culture water reaches up to  $10^6$  CFU.ml<sup>-1</sup> (FAO, 2014;

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Suantika et al., 2017; Suantika 2018b). The total *Vibrio* sp. population in the shrimp culture water in optimal conditions has been reported to be  $10^3$ – $10^5$  CFU.ml<sup>-1</sup> (Suantika et al., 2017, 2018a,b,c). However, opportunistic *Vibrio* may become pathogenic in specific environmental conditions, e.g. shrimp overcrowding stress in super-intensive aquaculture (Brock and Lightner, 1990).

The emergence of antibiotic-resistant strains of pathogenic bacteria has become a central issue in aquaculture, so alternatives for disease prevention and control need to be developed to address this emerging resistance (Smith, 2008). Formulating shrimp feed supplements to increase shrimp survival rate, growth, and disease resistance has – so far – been the most popular approach (Situmorang et al., 2020). In the last decade, the development of synbiotics, a mixture of pre- and probiotics, has been intensively studied for their potential application as feed supplements in *L. vannamei* culture (Oktaviana et al., 2014; Arisa et al., 2015; Huynh et al., 2018). Probiotics are live microbes which impart beneficial effects on host cells (Das et al., 2017). In *L. vannamei* culture, these benefits include the enhanced production of digestive enzymes in the shrimp gut, controlling the dominance of pathogenic bacteria, activating the intestinal immune system, and improving nutrient absorption (Zhou et al., 2009; Ramezani et al., 2014). Prebiotics are non-digestible food compounds that can selectively stimulate the growth or activity of beneficial bacteria in the digestive tract (Arisa et al., 2015). Several studies have shown that *L. vannamei* fed with prebiotic supplements show increased antimicrobial activity, enhanced phagocytic capacity, and less attachment of pathogenic bacteria in the gut (Anuta et al., 2014; Hardjani et al., 2017). Synbiotics alludes to synergism, and this term should be reserved for products in which the prebiotic selectively favor the probiotic organism(s) (Cencic and Chingwaru, 2010). Synbiotics were developed to overcome the possible survival difficulties for probiotics. A more efficient stimulating effect of the growth of probiotics and ubiquitous bacteria contribute on maintaining the intestinal homeostasis and a healthy body (Pandey et al., 2015).

Recently, the red seaweed *Kappaphycus alvarezii* has been studied as a potential prebiotic in the production of shrimp feed supplements (FAO, 2014; Suantika et al., 2017; Suantika 2018b). *K. alvarezii* is one of the main aquatic plants produced in aquaculture, with a global total production of 1.6 million tons in 2016 (FAO, 2018). *K. alvarezii* contains kappa-carrageenan, a potential immunostimulant which may improve shrimp immune response against pathogens (Hardjani et al., 2017; Suantika et al., 2017). However, this species lacks some amino-acid and essential fatty acid content and thus needs to be supplemented with other substances prior to its use as a shrimp feed supplement. The cyanobacteria *Spirulina* sp. can be used to complement *K. alvarezii*, as it contains 60–70% protein and the essential amino acids present are around 47% of total protein weight that are important in shrimp diets (Glencross et al., 2020; Ibrahim et al., 2013). The following amino acids required by shrimp for the optimal growth were provided in *Spirulina*: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine (Glencross et al., 2020).

Several probiotic bacteria have been investigated in *L. vannamei* diet supplementation, growth, and disease control (Zhang et al., 2009; Chandran et al., 2014). In shrimp culture,

*Halomonas* sp. is used as a probiotic and has been shown to increase shrimp resistance to the white spot syndrome virus (Zhang et al., 2009). In general, the probiotic products should have a minimum concentration of  $10^6$  CFU.ml<sup>-1</sup> or gram and a total of around  $10^8$ – $10^9$  probiotic microorganisms should be consumed daily for the probiotic effect to be transferred to the consumer (Kechagia et al., 2013). Our study aimed to investigate the effect of a dietary synbiotic containing *K. alvarezii* and *Spirulina* sp. prebiotics and local probiotic isolates of *H. alkaliphila* with optimal concentration for the survival, growth, and vibriosis resistance of post-larval shrimp culture.

## 2 Materials and methods

### 2.1 Probiotic production

*H. alkaliphila*, isolated from shrimp, was grown on Luria Bertani medium (Oxoid, Indonesia) (30, 25 °C) for 12 h. Bacterial cells were harvested through centrifugation (445 × g, 5 min, 25 °C); bacterial pellets were then rinsed using sterile distilled water to remove the remaining medium and freeze-dried (FD) using Freeze Dryer VD-800F (Taitec, Japan) for 24 h. The concentration of viable bacteria from the resultant powder after freeze-drying (CFU.gram FD<sup>-1</sup>) was measured using the total plate count (TPC) method with a spread technique on a nutrient agar medium (Oxoid, Indonesia) with salinity 30 (Cappuccino and Sherman, 2014). The amount (g of FD) of probiotic powder addition on feed (P) was calculated by:

$$P = \frac{X_t}{X_o}$$

where  $X_t$  was the final probiotic concentration in the feed (CFU) and  $X_o$  was the concentration of the bacterial powder (CFU.g FD<sup>-1</sup>).

### 2.2 Synbiotic feed production and formulation

Synbiotic feed was produced through the addition of synbiotic products into commercial (basal) feed. The commercial feed (Feng Li®, Indonesia) contained 41% protein, 7% lipid, 3% fiber, 13% ash, and 10% water. In this study, synbiotic feeds were produced by adding the prebiotic *K. alvarezii* (Helos®, PT. Gapura Akua Kultiva, Indonesia) at a concentration of 0.375% and *Spirulina* sp. (CERES®, Indonesia) at a concentration of 0.125% to the commercial feed, as well as *H. alkaliphila* powder at three different concentrations:  $10^8$  CFU.kg<sup>-1</sup> feed (S8 group),  $10^9$  CFU.kg<sup>-1</sup> feed (S9 group); and  $10^{10}$  CFU.kg<sup>-1</sup> feed (S10 group). The prebiotic feed (P group) was formulated by adding the prebiotic *K. alvarezii* and *Spirulina* sp. in the concentrations stated above, without *H. alkaliphila*. The commercial feed control (C group) was fed with 100% commercial feed without added synbiotics. Feed was stored in a dry place at room temperature (25 °C).

### 2.3 Proximate analyses

Analyses were performed at Saraswanti Indo Genetech (Bogor, Indonesia) to determine the total energy per biomass

and total energy from fat, as well as water, ash, total lipid, protein, and total carbohydrate content in all five diets. Ash content was measured by heating a sample to 600 °C until a constant weight was obtained (Situmorang et al., 2020). Protein content was measured using the Kjeldahl method (Maehre et al., 2018), and the lipid content was measured using the Weibull method (Situmorang et al., 2020). Carbohydrate content (including nitrogen-free extract and crude fiber) was calculated by subtracting the other elements from the total weight (FAO, 2014).

## 2.4 Experimental set-up and feeding test

A batch culture system using 15 10-L tanks equipped with aeration lines and water heaters and using seawater from Indramayu, Jawa Barat, Indonesia to culture shrimps. The seawater was sterilized using sodium hypochlorite (Bayclin®, Indonesia), then neutralized using sodium thiosulfate. Initial culture system conditions were as follows: salinity, 30–33; temperature, 30–32 °C; dissolved oxygen, 4–7 mg.L<sup>-1</sup> (Suantika et al., 2013). A total of 9750 specific pathogen-free (SPF) 5-day-old shrimp in the post-larval stage (PL5) with an average initial mean (±SD) body weight of 2.74 ± 0.23 mg and an average initial body length (±SD) of 0.64 ± 0.11 cm (random sampling from 20 shrimps) were stocked in 15 culture tanks at a high density of 650 PL per tank. All treatments were tested in triplicates over 14 days. Shrimp were fed four times a day 10% of their initial body weight and adjustments were made every day, assuming the shrimps gained 10% weight daily (Suantika et al., 2018a,b,c). During the experimental period, a 10% water renewal and debris/sediment siphoning were performed three times a week to maintain optimal water quality parameters. Water physicochemical parameters, including pH, temperature, dissolved oxygen (DO), and salinity level were measured every two days. NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> levels were also measured every two days using Nessler, diazotization, and nitrate HCl methods (Suantika et al., 2018a,b,c). The synbiotic treatment enabling the best shrimp survival was selected to undergo a bacterial challenge test using opportunistic pathogen *V. harveyi*.

## 2.5 *Vibrio harveyi* production and challenge test

After 14 days of feeding test, a bacterial challenge test for the C, P, and S9 treatment groups was performed for 7 days using *V. harveyi* obtained from Balai Besar Perikanan Budidaya Air Payau Jepara, Central Java, Indonesia. *V. harveyi* was cultured in Luria Bertani Broth medium (Oxoid, Indonesia) (salinity 30, temperature 25 °C) for 12 h. After incubation, the density of the bacterial culture was determined by measuring turbidity with a spectrophotometer (Genesys 20, Termospectronic) at a wavelength of 550 nm. *V. harveyi* culture (10 ml) at a concentration of 10<sup>10</sup> CFU.ml<sup>-1</sup> was harvested by centrifuging at 89 × g for 10 min and washed twice in its culture medium, then washed once using shrimp culture water before being added to each respective tank. The final concentration of *V. harveyi* in the tank was 10<sup>6</sup> CFU.ml<sup>-1</sup>. For each experimental feed in the challenge test, a non-exposed group without *Vibrio* (non-challenged) was tested

and compared to the groups infected with *Vibrio*. Each treatment was performed in triplicates over seven days.

## 2.6 Bacterial enumeration of shrimp and culture water samples

Three post-larval shrimps were collected from each tank at day 0, 7, and 14 during the feeding test, euthanized in an ice-cold 5 g L<sup>-1</sup> benzocaine solution (Sigma, E-1501), then rinsed with sterile seawater. Shrimp samples from the same tank were pooled and homogenated using a micro pestle with sterile NaCl 0.85% in a 1.5-ml microtube. Additionally, 50 ml of culture water was also collected from each tank. Shrimp homogenate was diluted 100× using NaCl 0.85%; the culture water sample was similarly diluted. The number of heterotrophic bacteria in shrimp and water sample were recorded using the total plate count (TPC) method with spread technique using 100 µL of diluted shrimp gut homogenate on natrium agar medium (Oxoid, Indonesia) with salinity 30, respectively. Inoculated agar plates were incubated at room temperature (25 °C) for 24 h before the number of grown colonies was counted (Cappuccino and Sherman, 2014).

The initial and final concentration of *Vibrio* sp. in water and shrimp cultures from the challenged groups also was measured using the TPC method with a spread technique on thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium (Oxoid, Indonesia) with salinity 30 (Cappuccino and Sherman, 2014).

The Shannon-Weaver index to measure bacterial diversity in shrimp and culture water samples was calculated using the following formula (Lemos et al., 2011):

$$H = - \sum_{i=1}^s (p_i \ln p_i)$$

where  $s$  is the number of operational taxonomic unit (OTUs) and  $p$  is the proportion of the community represented by OTU  $i$ . The Shannon-Weaver index estimates species richness species evenness within a given sample (Lemos et al., 2011).

## 2.7 Bacterial identification

The dominant bacterial culture observed from shrimp culture during feeding test was purified and identified by bacterial DNA extraction, polymerase chain reaction amplification (27F/1492R primer), and bacterial rRNA 16S gene sequencing (785F/907R primer) at Macrogen, Korea. The sequence homology search for test sequences was performed using Nucleotide Blast versus GenBank and Ribosomal Database Project data. Results were validated via a phylogenetic analysis using the Winclada program and the Ratchet method (Island Hopper) (Suantika et al., 2017).

## 2.8 Growth and survival measurements

The average survival percentage, weight gain, and length increase following the 14-day feeding experiment and 7-day challenge test of each treatment groups were calculated

**Table 1.** Proximate analysis results of commercial feed (C), probiotic-supplemented feed (P), synbiotic-supplemented feed using *Halomonas alkaliphila*  $10^8$  CFU.kg<sup>-1</sup> (S8), *Halomonas alkaliphila*  $10^9$  CFU.kg<sup>-1</sup> (S9), and *Halomonas alkaliphila*  $10^9$  CFU.kg<sup>-1</sup> (S10) used in this experiment, with the optimum range values provided for comparison.

Parameter	Optimum range <sup>a</sup>	C	P	S8	S9	S10
Total energy (kcal.100 g <sup>-1</sup> )	223–371	359.70	356.84	359.02	360.68	359.64
Energy from fat (kcal.100 g <sup>-1</sup> )	52–70	78.80	74.52	77.58	78.84	78.48
Water content (%)	7–12	9.00	9.08	8.92	8.94	9.11
Ash content (%)	<16	12.20	12.06	12.08	11.84	11.88
Total fat (%)	6.50–11.70	8.76	8.28	8.62	8.76	8.72
Protein (%)	40–50	35.55	35.80	35.5	35.16	35.66
Carbohydrate (%)	20–40	34.68	34.78	34.86	35.30	34.63

<sup>a</sup>Optimum range nutrient requirement information derived from Davis (2005).

as follows:

$$\text{Survival (\%)} = \frac{\text{Number of shrimp at the end of culture period}}{\text{Number of shrimp at day 0}} \times 100$$

$$\text{Weight gain (g)} = \text{weight at the end (g)} - \text{weight at day 0 (g)}$$

$$\text{Length increase (cm)} = \text{length at the end (g)} - \text{length at day 0 (g)}$$

## 2.9 Statistical analysis

Survival data were first normalized using an arcsine transformation before statistical analysis (Suantika et al., 2018a,b,c). To evaluate the differences between treatment groups, data on growth and survival parameters were analyzed using one-way ANOVA and followed by Duncan posthoc Test with 95% confidence intervals. All statistical analyses were performed using SPSS<sup>®</sup> Version 24.0.

## 3 Results

### 3.1 Probiotic concentration and effect of its addition into the experimental feed

The probiotic powder was produced through the freeze-drying method, a commonly used technique for the production of dried probiotics. Cells are first frozen to below the critical temperature of the formulation, then dried by sublimation under high vacuum in two phases: primary drying, during which unbound water is removed; and secondary drying, during which the bound water is removed (Savani et al., 2010; Jalali et al., 2012). The TPC in the freeze-dried bacterial powder showed that viable *H. alkaliphila* was found at a concentration of  $2.0 \times 10^{10}$  CFU.g FD<sup>-1</sup>. Three different synbiotic feeds were made by adding 5.19, 51.9, and 518.7 mg of freeze-dried *H. alkaliphila* into commercial feed for S8, S9 and S10, respectively.

### 3.2 Proximate dietary analysis

The percentages of each component from the three different diets used in this study are presented in Table 1.

**Table 2.** The effect of the 14-day feeding test on *Litopenaeus vannamei* using control commercial feed (C); probiotic-supplemented feed (P); and synbiotic-supplemented feed with *Halomonas alkaliphila*  $10^8$  CFU.kg<sup>-1</sup> (S8),  $10^9$  CFU.kg<sup>-1</sup> (S9), and  $10^{10}$  CFU.kg<sup>-1</sup> (S10).

Code	Survival (%)	Weight Gain (mg)	Length Increased (cm)
C	65.70 ± 0.01 <sup>a</sup>	3.10 ± 1.10 <sup>a</sup>	0.23 ± 0.12 <sup>a</sup>
P	78.00 ± 0.02 <sup>b</sup>	3.30 ± 1.20 <sup>a</sup>	0.30 ± 0.33 <sup>a</sup>
S8	71.70 ± 0.11 <sup>ab</sup>	4.70 ± 0.85 <sup>a</sup>	0.31 ± 0.13 <sup>a</sup>
S9	91.50 ± 0.05 <sup>c</sup>	5.60 ± 1.89 <sup>a</sup>	0.34 ± 0.14 <sup>a</sup>
S10	66.20 ± 0.03 <sup>ab</sup>	4.80 ± 1.58 <sup>a</sup>	0.33 ± 0.13 <sup>a</sup>

Generally, the synbiotic-supplemented feed contained a higher amount of carbohydrate compared to commercial feed (i.e., the control), while the S9 diet had the highest total energy (360.68 kcal.100 g<sup>-1</sup>).

### 3.3 Water quality parameters

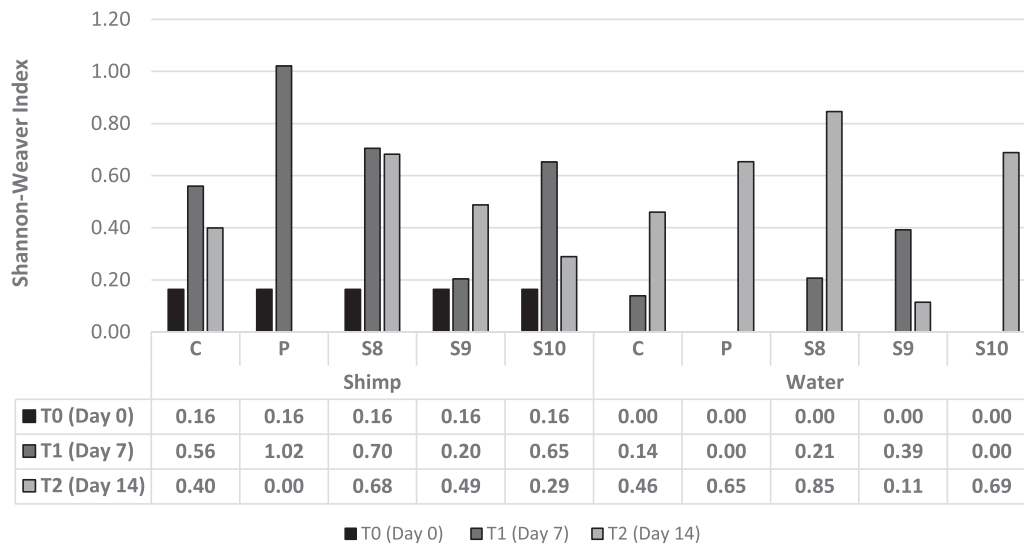
All water quality parameters were in the optimum range for shrimp cultivation (Suantika et al., 2018a,b,c), including dissolved oxygen (4.0–6.0 mg.L<sup>-1</sup>), pH (7.0–8.5), temperature (29–32 °C), salinity (30–35), ammonium (0.01–0.15 mg.L<sup>-1</sup>), and nitrite (0.1–1.1).

### 3.4 Analysis of shrimp biological parameters in feeding test

Following the 14 days culture period, the survival of *L. vannamei* in the synbiotic (S9) and probiotic (P) group was significantly higher than the control group ( $p < 0.05$ ). The survival S8 and S10 (71.7% and 66.2%, respectively) were not significantly different from C (65.7%) or P (78%;  $p > 0.05$ ; Tab. 2). The highest survival rate was 91.5% in the S9 group, which was significantly higher than the control (survival rate: 65.7%;  $p < 0.05$ ). No significant differences in the changes in average body weight (+3–6 mg) and length (+0.23–0.34 cm) were detected between the treatment groups (Tab. 2). The S9 and P groups were subsequently be used in the challenge test

**Table 3.** Results of the 7-day challenge test against *Vibrio harveyi* on post-larval *Litopenaeus vannamei* with control commercial feed (C), prebiotic-supplemented feed (P), and synbiotic-supplemented feed with *Halomonas alkaliphila*  $10^9$  CFU.kg<sup>-1</sup> (S9).

	Code	Survival (%)	Weight gain (mg)	Length increased (cm)
Unchallenged group	C	65.50 ± 0.02 <sup>a</sup>	3.50 ± 2.15 <sup>a</sup>	0.20 ± 0.15 <sup>a</sup>
	P	80.00 ± 0.03 <sup>b</sup>	3.00 ± 2.07 <sup>a</sup>	0.20 ± 0.14 <sup>a</sup>
	S9	83.30 ± 0.05 <sup>b</sup>	4.50 ± 2.26 <sup>a</sup>	0.20 ± 0.09 <sup>a</sup>
	CV	39.50 ± 0.04 <sup>c</sup>	2.70 ± 1.36 <sup>a</sup>	0.10 ± 0.09 <sup>a</sup>
Challenged group	PV	56.60 ± 0.05 <sup>d</sup>	1.90 ± 1.46 <sup>a</sup>	0.20 ± 0.08 <sup>a</sup>
	S9V	79.90 ± 0.05 <sup>b</sup>	4.00 ± 2.70 <sup>a</sup>	0.20 ± 0.09 <sup>a</sup>

**Fig. 1.** The Shannon-Weaver index of the bacterial community in shrimp culture and water culture water during the feeding test at day 0 (T0), day 7 (T1), and day 14 (T2) during the feeding test using commercial feed (C), prebiotic-supplemented feed (P), synbiotic-supplemented feed using *Halomonas alkaliphila*  $10^8$  CFU.kg<sup>-1</sup> (S8), *Halomonas alkaliphila*  $10^9$  CFU.kg<sup>-1</sup> (S9), and *Halomonas alkaliphila*  $10^9$  CFU.kg<sup>-1</sup> (S10).

against *V. harveyi* and compared to the C treatment group as a control.

### 3.5 Shrimp biological parameters in the challenge test against *V. harveyi*

In the challenge test, the highest survival of *L. vannamei* was obtained by the S9 group in both non-challenged and challenged conditions ( $p < 0.05$ ). The survival of *L. vannamei* in the P and C groups significantly decreased with the addition of a lethal dose of *V. harveyi* (i.e.,  $10^6$  CFU.ml<sup>-1</sup>;  $p < 0.05$ ). However, there was no reduction of shrimp survival in the S9 group or synbiotic treatment even in the post-challenge test. In general, no significant differences in changes in average body weight (+0.02–0.05 g) and length (+0.15–0.25 cm) were found between treatments groups (Tab. 3).

### 3.6 Analysis of dominant bacterial colonies during the feeding test

The Shannon-Weaver index in the synbiotic treatment was higher than that of the control, suggesting its bacterial community was diverse and abundant (Fig. 1). Pre-dominant

bacterial isolates showed differences between sampling time points and between treatments. The synbiotic treatment resulted in more variation in the diversity of dominant bacterial isolates in both the water and shrimp samples, including species like *Bacillus cereus*, *Pseudoaeromonas piscicida*, and *Meridianimaribacter flavus* (Fig. 2).

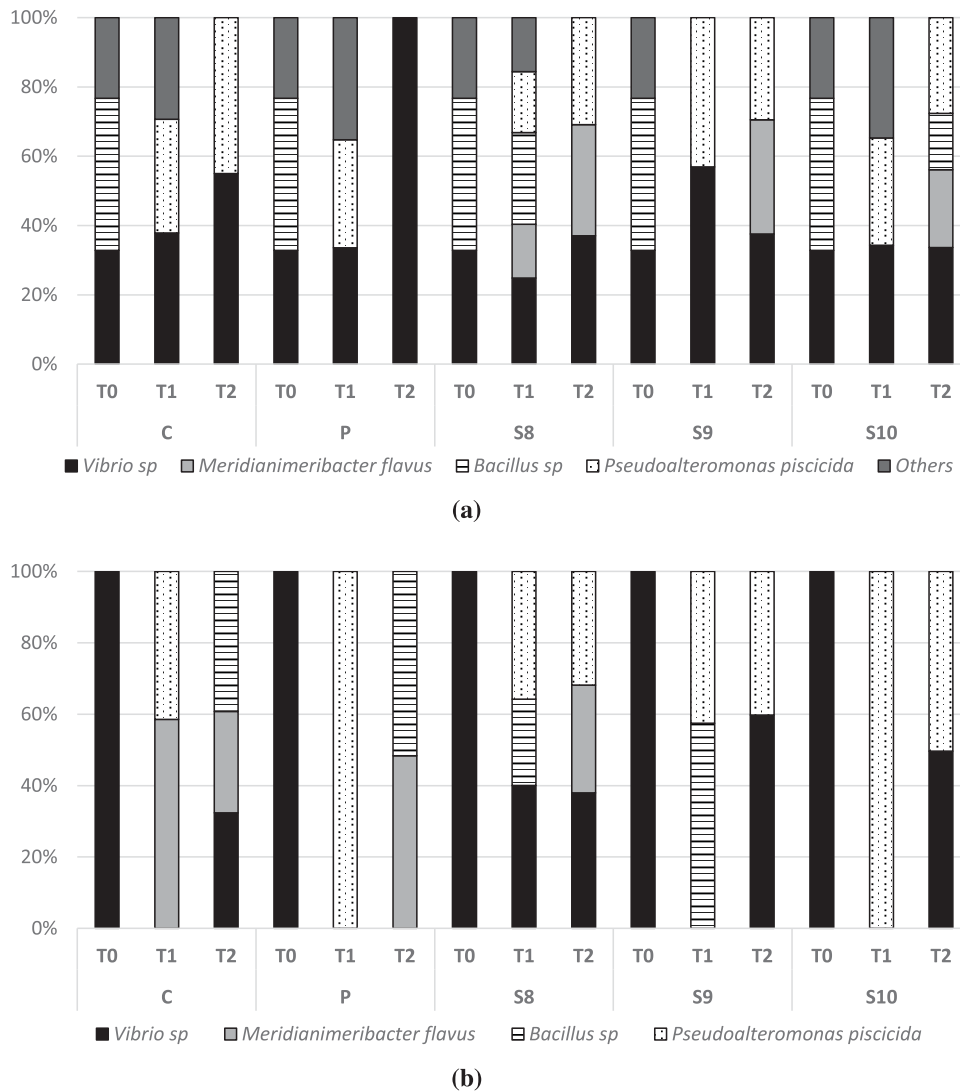
### 3.7 Concentration of *Vibrio* sp. in the challenge test

In general, *Vibrio* sp. in cultivation systems reaches the lethal dose for shrimps at  $\sim 10^6$  CFU.ml<sup>-1</sup>. The prebiotic and synbiotic treatments can reduce the total concentration of *Vibrio* sp. in both the shrimp and water samples; however, the synbiotic treatment was the most effective in this regard: achieving a reduction from  $10^6$  CFU.ml<sup>-1</sup>– $10^2$  CFU.ml<sup>-1</sup> in culture water (Tab. 4).

## 4 Discussion

### 4.1 Proximate analysis

The addition of *H. alkaliphila* was found to increase the carbohydrate content in the synbiotic feed. According to



**Fig. 2.** Percentage of dominant bacterial community in shrimp culture (a) and water culture (b) at day 0 (T0), day 7 (T1), and day 14 (T2) during the feeding test using commercial feed (C), prebiotic-supplemented feed (P), synbiotic-supplemented feed using *Halomonas alkaliphila*  $10^8$  CFU.kg<sup>-1</sup> (S8), *Halomonas alkaliphila*  $10^9$  CFU.kg<sup>-1</sup> (S9), and *Halomonas alkaliphila*  $10^9$  CFU.kg<sup>-1</sup> (S10).

**Table 4.** The total number of *Vibrio* in the water sample and shrimp samples at the initial and the end of the culture period in the challenge test when treated with control commercial feed (CV), prebiotic feed (PV), and synbiotic feed (S9V).

	Code	Total <i>Vibrio</i> sp. Number (CFU.ml <sup>-1</sup> )	
		Initial (Log 10)	End (Log 10)
Shrimp culture	CV	$2.0 \times 10^3$	$7.0 \times 10^3$
	PV	$3.4 \times 10^4$	$7.6 \times 10^3$
	S9V	$3.4 \times 10^4$	$6.6 \times 10^3$
Culture water	C	$5.0 \times 10^4$	$1.2 \times 10^5$
	PV	$1.4 \times 10^6$	$3.3 \times 10^4$
	S9V	$1.0 \times 10^6$	$2.0 \times 10^2$

*K. alvarezii* contains polysaccharides that also can increase the feed carbohydrate content (Suantika et al., 2017).

#### 4.2 Water quality in the shrimp culture system

During performance and challenges test, the physicochemical properties of the culture system; including dissolved oxygen, pH, temperature, salinity, as well as ammonium, nitrite, and nitrate levels remain in the optimum range for shrimp cultivation. Hence, the water quality has no negative effect on the survival and growth of *L. vannameii*.

#### 4.3 Biological parameters of *L. vannameii* after the feeding and challenge tests

Synbiotic feed supplementation resulted in beneficial effects for *L. vannameii* post-larval cultures in this study. The higher survival and vibriosis resistance even after the

Aquacop (1978), bacteria can contribute to the bulk of carbohydrate, especially when shrimp are reared in an earthen pond or super-intensive conditions. Additionally, the

challenge test against *V. harveyi* indicates that the probiotics and prebiotics in the tested synbiotics acted synergistically. Hypothetically, the mechanism behind this effect is complex: first, *K. alvarezii* is used as a nutritional source for probiotic bacteria, which enhances their growth while limiting that of opportunistic and potentially pathogenic bacteria. Then, the halophilic *H. alkaliphila* produces the enzyme alpha-glucosidase (Romano et al., 2006), which suggests that *H. alkaliphila* can potentially hydrolyze oligosaccharides and polysaccharides (non-digestible carbohydrates) in *K. alvarezii*. The metabolic results of non-digestible carbohydrates can be in the form of short-chain fatty acids (SCFA), which can stimulate the activation of the immune system (Morrison and Preston, 2016). *Halomonas* sp. are also through to cause *Vibrio* quorum quenching. The lactonase produced by *Halomonas* sp. can hydrolyze N-acylhomoserine lactone (AHL) which is an autoinducer in *Vibrio* quorum sensing (Romano et al., 2006; Fangfang et al., 2008).

*Vibrio* spp. Are natural opportunistic micro-flora of shrimps (Sindermann, 1990) and may become pathogenic when shrimps are in sub-optimal condition, such as physiological unbalance and nutritional deficiency, or when there are other stressors, i.e., poor water quality and overstocking, which allow opportunistic bacterial infections to progress (Brock and Lightner, 1990). Indeed, inappropriate levels of shrimp larval density and poor living conditions of shrimp larvae may also cause *Vibrio* to become pathogenic (Brock and Lightner, 1990). The shrimp density used in this study was very high compared to the common shrimp stocking density of 60–300 PL.m<sup>-3</sup> (FAO, 2009; Suantika et al., 2018a,b,c, 2020). In normal conditions, high stocking density may affect shrimp survival, because the *Vibrio* population can be pathogenic in the batch shrimp culture system. This might in turn result in high shrimp mortality (up to 70%), even in treatment groups without *Vibrio* addition. However, the use of synbiotic feed still provided the highest shrimp survival, suggesting it also increased vibriosis resistance.

#### 4.4 Bacterial community during feeding test and challenge test

The use of synbiotic feed is known to induce the growth of beneficial intestinal bacteria (Pandey et al., 2015). In this study, *P. piscicida* and *B. cereus* isolated from shrimp and culture water samples subjected to the synbiotic treatment could be used as a probiotic to increase disease resistance (Far et al., 2009; Ramadhani et al., 2019). *Vibrio* quorum quenching in shrimp and culture water can be possible due to synbiotic-feed treatment, so the pathogenicity of *Vibrio* will decrease. Lactonase may have produced by *H. alkaliphila* which can hydrolyze AHL (N-acylhomoserine lactone) as autoinducer in *Vibrio* quorum sensing (Romano et al., 2006; Fangfang et al., 2008). In this study, the increased of beneficial bacteria in synbiotic treatment may also reduce the dominance of *Vibrio*.

## 5 Conclusion

In conclusion, supplementing the commercial feed used in shrimp aquaculture with *K. alvarezii* and *Spirulina* sp.

prebiotics and *H. alkaliphila* probiotic enhance the survival of *L. vannamei* in post-larval cultures, under both normal conditions and when infected with *V. harveyi*. This suggests that the tested synbiotic feed can potentially be used as a vibriosis biocontrol agent in intensive shrimp aquaculture.

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