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Innovation of Sinbiotic Formula for the Growth of White Shrimp Larvae (*Litopenaeus vannamei*)

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Abstract

In the hatchery of white shrimp larvae, there are some problems, such as a decreased water quality and diseas-es caused by bacteria followed by decrease of growth. The solution to reduce these problems is by applying a formula of synbiotics. Synbiotic is a combination of probiotics and prebiotics in a form of synergism. This re-search aimed to make an innovative synbiotic formula for the absolute length growth and survival rate of white shrimp larvae (Litopenaeus vannamei) as well as the total number of bacteria and the number of Vibrio sp. In this study, there were six treatments, as follows: C-: negative control (without synbiotic application) C+: posi-tive control (commercial synbiotic) P: probiotic 2.5 x 10^5 cfu/ml and prebiotic 1.5 ppm Q: probiotic 5 x 10^5 cfu/ml and prebiotic 1 ppm R: probiotic 7.5 x 10^5 cfu/ml and prebiotic 0.5 ppm S: probiotic 106 cfu/ml and prebiotic 2 ppm, each treatment with four repetitions. This research was set up in a completely randomized de-sign experiment using twenty-four plastic tanks with 50 L total volume filled with 20 L sterile sea water and stocked with 4.000 nauplii in the PT. Citra Larva Cemerlang hatchery, Kalianda, Lampung. Variables ob-served in this research are survival rate, absolute length growth, total bacterial and Vibrio sp. counts, and wa-ter quality in the white shrimp larvae (Litopenaeus vannamei). Results show that the best survival rate is in treatment S (probiotic 106 cfu/ml and prebiotic 2 ppm) (87.7%), the highest absolute length growth is in treat-ment P (3.8 mm), the highest total bacteria was in treatment S (11.1 log cfu/ml), and the best total Vibrio sp. is in treatment S (3.5 log cfu/ml). Water quality of the six treatments shows results that were still in normal condi-tions following Indonesian National Standard SNI 7311: 2009.

Keywords: Litopenaeus vannamei*; absolute length growth; synbiotic; survival rate*

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1. Introduction

White shrimp *(Litopenaeus vannamei)* is one of Indonesia's superior fishery commodities because it has high economic value and productivity. White shrimp production in Indonesia recorded reaches 6-10 tons/ha/year and continues to increase every year [1].

The white shrimp hatchery has some various problems, such as a decrease in water quality and diseases caused by bacteria that cause a decrease in shrimp growth. The solution to reduce these problems is by applying probiotics, prebiotics, or synbiotics. Probiotics are living microorganism that are beneficial to their host. Several mechanisms of action of probiotic bacteria in inhibiting the presence of pathogenic bacteria include producing inhibitory compounds, competition for attachment sites, competition for nutrients sources, and improving water quality [2]. Prebiotics are organic compounds that can be used as nutrients for probiotic bacteria but cannot be digested by probiotic hosts. The role of probiotics can be increased by the use of prebiotics because prebiotic compounds can stimulate the growth and activity of certain bacteria in the intestine, thereby improving the health of the host [3].

In addition to probiotics and prebiotics as those compounds in balances synbiotics can also support growth in shrimp. Synbiotic are a combination of probiotics and prebiotics in balance. The mechanism of synbiotic is to control the number of microorganism in the digestive tract. A good combination of prebiotics and probiotics as synbiotics can increase the survival of probiotic bacteria because specific substrates already exist for fermentation. As the results of Damayanti's research which states that synbiotics can improve the immune response and increase survival by up to 80% and growth rates to 7.59% in shrimp [4].

Therefore, this research aimed to find a synbiotic formula as innovative solution for the absolute length growth and survival rate of white shrimp larvae *(Litopenaeus vannamei)* as well as total number of bacteria and the number of *Vibrio* sp.

2. Materials and Methods

2.1 Synbiotic Preparation

The synbiotic used is the mixture of *Bacillus* sp. IBK3 [5], *Bacillus* sp. UJ131 [6], *Lactobacillus* sp., and Anoxygenic Photosynthetic Bacteria (APB), as well as oligosaccharides extracted from yam *(Pachyrhizus erosus)* flour. The *Bacillus* sp. IBK3 is mannanolityc isolate [7]. The *Bacillus* sp. UJ131 are proteolytic, cellulolytic and xylanolytic isolate [8]. Before being used, probiotic bacteria were cultured using agar media. *Bacillus* sp. and Anoxygenic Photosynthetic Bacteria (APB) were cultured using *Sea Water Complete* (SWC) agar, and *Lactobacillus* sp. were cultured using *deMan, Rogosa and Sharpe* (MRS) agar. After that, *Bacillus* sp. cultures were inoculated to *Sea Water Complete* (SWC) *broth, Lactobacillus* sp. cultures were inoculated to *Sea Water Complete* (SWC) *broth*, and Anoxygenic Photosynthetic Bacteria (APB) cultures were inoculated to *deMan, Rogosa and Sharpe* (MRS) *broth.* After that, bacterial density was calculated up to 10^9 sel/mL. The calculation of bacterial density was done by a direct method under a microscope. Then, probiotic bacteria were made to large-scale bacterial stock. *Bacillus* sp. was cultured in *Nutrient Broth*, while *Lactobacillus* sp. was cultured in *Sea Water Complete* (SWC) *broth*, and Anoxygenic Photosynthetic Bacteria (APB) was cultured in *deMan, Rogosa and Sharpe* (MRS) *broth*. Bacterial cultures were stored at 4^{o}C to maintain their viability.

To get oligosaccharides from yam, yam flour was made based on Sukenda's research method [9]. At first, the yam was peeled and cleaned, then the yam was sliced using a slicer with a thickness of ± 1 mm. The yam slices were dried in an oven at 55^{o}C for 5 hours until the yam slices could be broken by hand easily. Yam slices were mashed and sifted. Then the resulting yam flour was produced. The next step was oligosaccharides extraction from the yam flour. First, yam flour was suspended in 70% ethanol with a ratio of 1:10 and stirred at room temperature and the residue is washed with 70% ethanol. Next, it was precipitated and filtered using filter paper and sterile funnel. The filtrate was concentrated using a rotary evaporator at 40^{o}C to eliminate the ethanol content and preserve the oligosaccharide extract from the yam flour, the extract was stored in an oven at 40 ^{o}C until it becomes a paste. Then, prebiotic in a form of paste could be applied to white shrimp larvae. To find out the concentration of oligosaccharides contained in the yam paste, a sugar reduction test was performed using the DNS method.

2.2 In Vivo Synbiotic Application

This research was conducted for 24 days from 30 March - 22 April 2020 at PT. Citra Larva Cemerlang, Kalianda Lampung. This study was conducted in a completely randomized design (CRD) with six treatments and four repetitions, which included four types of synbiotic concentrations and control for comparison. The controls used were negative and positive controls (Table 1).

Table 1. Test Treatments on White Shrimp Larvae (*Litopeneus vannamei*)

Treatments	Explanation
C-	Negative control (without synbiotic application)
C+	Positive control (commercial synbiotic)
P	Probiotic 2.5 x 10^5 cfu/ml and prebiotic 1.5 ppm
Q	Probiotic 5.0 x 10^5 cfu/ml and prebiotic 1 ppm
R	Probiotic 7.5 x 10^5 cfu/ml and prebiotic 0.5 ppm
S	Probiotic 10 x 10^5 cfu/ml and prebiotic 2 ppm

Research containers used in this research are 24 units' tanks with 50 liters optimum volume and aerated. Containers and aerator equipment had been sterilized using chlorine with a concentration of 150 ppm. The media used was sea water with 30 ppt salinity, aerator and heater were set without calculating the aerator strength to maintain sea water temperature (30 ± 1^{o}C). Each container filled with 20 L of sea water, then ADTA was added to the tanks to precipitate the remaining dirt and sterilization of pathogen microorganisms. Test animals used were 96,000 nauplii white shrimp *(Litopenaeus vannamei)* (4,000 nauplii/container). After being transferred to the test container, the test animals were acclimatized first, kept for one day until the zoea-1 stage without being fed and without any synbiotic treatments. White shrimp larvae started to be fed and treated with synbiotic treatment when they were zoea-1 stage. Feeding to white shrimp using natural feed and commercial feed, following the standard operating procedures (SOP). Synbiotic was applied directly through the water media and the time of application was following the Standard Operating Procedure (SOP). The test was carried out *in vivo* and white shrimp larvae were reared and treated for 17 days from zoea-1 to post larva-8.

Figure 1. White Shrimp Larvae (*Litopenaeus vannamei*)

2.3 Variables

After 17 days of rearing, survival rate, absolute length growth, total bacterial, and total *Vibrio* sp. counted in white shrimp *(Litopenaeus vannamei)* were calculated. The survival rate was calculated by the percentage difference between live shrimp before the treatment had been given and live shrimp after the treatment was given. Absolute length growth was calculated by measuring the difference between the average lengths of post-larvae-8 white shrimp at the end of the test (mm) with the average length of post-larvae-1 white shrimp (mm). Total bacterial and total of *Vibrio* sp. Counted by using the TPC (Total Plate Count) method.

Water quality testing in the form of temperature, pH, and salinity measurements is carried out every day during rearing. Temperature measurements were calculated by using a thermometer, pH was calculated by using pH meter, and salinity measurements were calculated by using a refractometer [10]. For the measurement of ammonia (NH3) levels were calculated using the Ammonia Medium Range Checker HC - HI715. Measurement of ammonia levels was done by following the Standard Operating Procedure (SOP), 3 times during the rearing period, the zoea-1 stage before the application of the synbiotic treatment, post-larvae-1, and post larvae-8 before harvesting.

3. Results and Discussion

3.1 Analysis of Oligosaccharide Concentrate in Yam Prebiotics

The DNS method was used because it is a commonly used method for the measurement of reducing sugars. The principle of the DNS method is 3,5 dinitrosalicylic acid as an oxidizer reducing sugars and produce 3-amino-5-dinitrosalicylic acid. The aldehyde group in the polysaccharide chain has been oxidized to a carboxyl group, at the same time the sugar aldehyde group will reduce the dinitrosalicylic acid. The reaction will cause a change of the color in yellow to reddish-orange. The reaction will continue as long as there is reducing sugar in the test solution [11].

In 1 gram of yam paste contained 0.285 grams of reducing sugar (28.5%) that is very effecient to use as prebiotic [16].

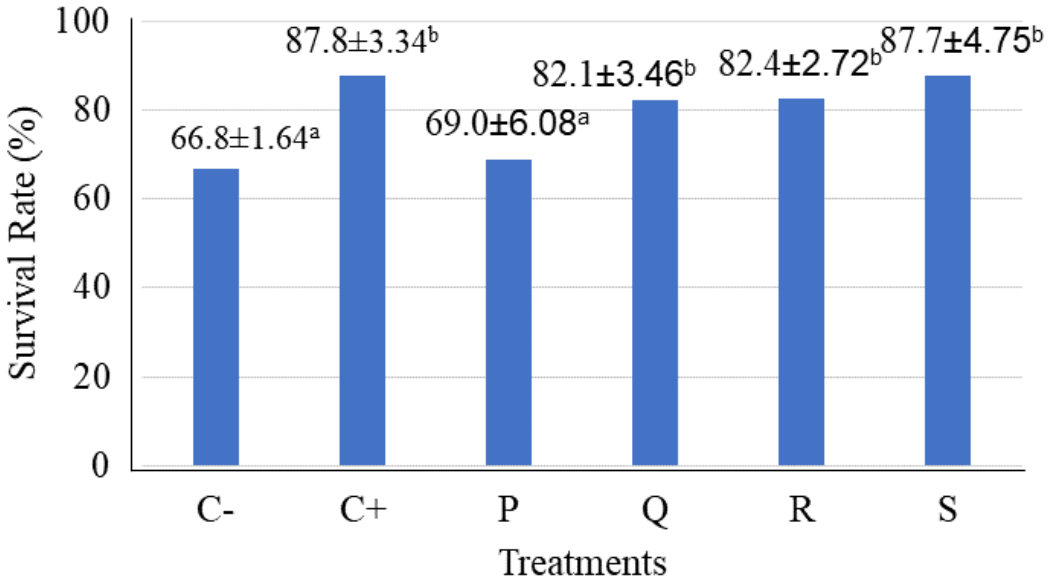

Figure 2. Survival Rates of White Shrimp Larvae Reared In Plastic Tanks with Different Doses of Synbiotic

C- = negative control (without synbiotic application); C+ = positive control (commercial synbiotic); P = probiotic 2.5 x 10^5 cfu/ml and prebiotic 1.5 ppm; Q = probiotic 5.0 x 10^5 cfu/ml and prebiotic 1 ppm; R = probiotic 7.5 x 10^5 cfu/ml and prebiotic 0.5 ppm; S = probiotic 10^6 cfu/ml and prebiotic 2 ppm

3.2 Effects of Synbiotics Application on Survival Rate of White Shrimp Larvae

The result of the survival rate of white shrimp larvae is presented in Figure 2 below. The highest survival rate is treatment S (87.7%). There are significant differences between treatment S and treatment C- (87.7% and 66.8%) ($P<0.05$). This proves that the best synbiotic dose is the mixture of 10^6 cfu/ml probiotics and 2 ppm prebiotics. Provision of probiotic bacteria with a density of 10^6 cfu/ml is done by following the Regulation of the Director General of Aquaculture No. 25/PER-DJPB/2016 about Guidelines for Testing the Quality of Fish Medicines which mentions the maximum bacterial content of 5 species with a density of 10^6 cfu/ml in minimum [12].

The provision of *Bacillus* sp. with a density of 10^6 cfu/ml as a probiotic increase the survival rate of white shrimp up to 92% [13]. Likewise, with the provision of probiotic bacteria, *Lactobacillus* sp. can increase the SR value of shrimp by 86.67% [14]. Darmawan's research showed that giving anoxygenic photosynthetic bacteria as probiotics could affect the survival rate of white shrimp larvae as 69.6% [15]. Meanwhile, according to the research of Sabila, it showed that giving oligosaccharide extract from yam could affect the survival rate of white shrimp larvae as 75% [16].

The effect of synbiotic administration on Q and R treatment still shows a good survival rate, Widig-do's stated that the survival rate categorized as good if the SR value is > 70%, survival rate categorized as a medium if the SR value is 50-60%, and survival rate categorized as low if the SR value is <50% [17].

3.3 Effects of Synbiotic Application to Absolute Length Growth of White Shrimp Larvae

Absolute length growth in treatment P ang C- has no significant differences (3.8 mm and 3.3 mm) ($P>0.05$). However, there are significant differences ($P<0.05$) of treatment P and C+ (3.8 mm and 2.9 mm). The highest and lowest absolute length growth are treatment P, C-, Q, R, S, and C+ (Figure 3). The length of shrimp larvae is inversely proportional to the density of shrimp larvae in the rearing tanks,

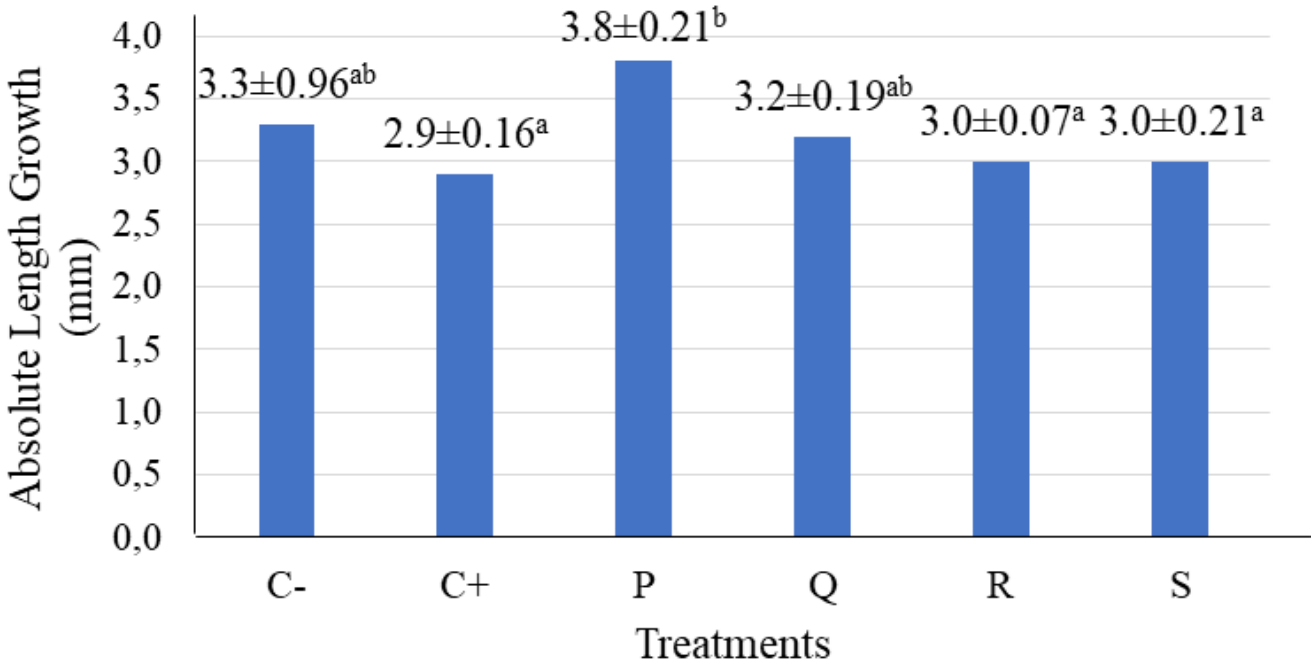

Figure 3. Absolute Length Growth of White Shrimp Larvae Reared in Plastic Tanks with Different Doses of Synbiotic

C- = negative control (without synbiotic application); C+ = positive control (commercial synbiotic); P = probiotic 2.5 x 10^5 cfu/ml and prebiotic 1.5 ppm; Q = probiotic 5.0 x 10^5 cfu/ml and prebiotic 1 ppm; R = probiotic 7.5 x 10^5 cfu/ml and prebiotic 0.5 ppm; S = probiotic 10^6 cfu/ml and prebiotic 2 ppm

presumably of the competition among shrimp larvae to get food in the tank. The less the density of shrimp larvae in the rearing tank (treatment C-), the more shrimp get nutrition so that the white shrimp larvae in the rearing tank of treatment C- will have the longer long gap compared by the other treatments.

High density in a rearing place could create a competition of shrimps for feed and dissolved oxygen. In a rearing tank with a high survival rate of white shrimp, the available feed and nutrients would be be divided into more shrimps so that each shrimps will get limited nutrition and feed and would cause the length of the shrimp to not be increased. Otherwise, if the survival rate in a white shrimp rearing thank was low, then the competition between shrimp for nutrition and feed was not as big as in the rearing tank with a high survival rate of white shrimps so that each shrimp would get more nutrition and feed, causing the length to increase more.

3.4 Effects of Synbiotic Application to Total Bacteria in the Rearing Water

The total bacteria is directly proportional to the probiotic bacteria given (Figure 4). The more the density of probiotic bacteria is given, the more total bacterial density is calculated at the end of rearing. Treatment C- is not significantly different with treatment P (10.6 log cfu/ml and 10.6 log cfu/ml) ($P>0.05$). However, treatment S is significantly different with treatment C- (11.1 log cfu/ml and 10.6 log cfu/ml) ($P<0.05$). The highest to lowest total bacteria are treatment S, C+, R, Q, C-, dan P. In treatment C+ or commercial synbiotics used, contains 5 species of microorganisms, including *Bacillus subtilis*, *Bacillus licheniformis*, *Lactobacillus acidophilus*, *Peanibacillus pumilus*, and *Saccharomyces cerevisae*. Whereas in treatment S, the bacteria used were 4 species of bacteria, *Bacillus* IBK3, *Bacillus* UJ131, *Lactobacillus* sp., and Anoxygenic Photosynthetic Bacteria. However, in the enumeration total bacteria results that

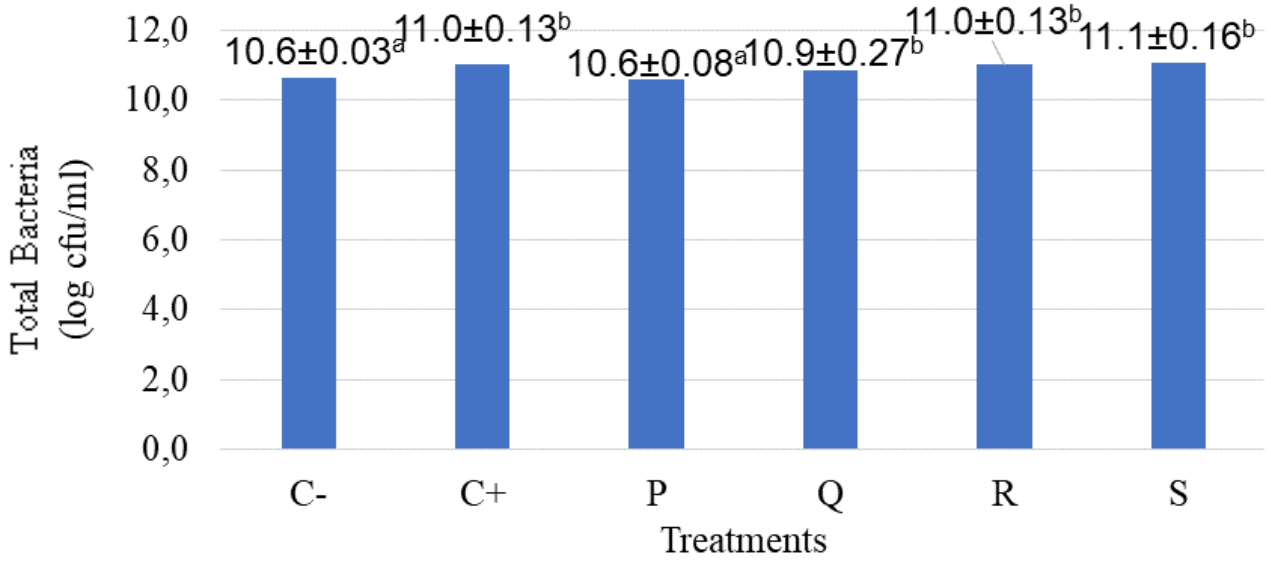

Figure 4. Total Bacteria in the Water of Rearing Plastic Tanks with Different Doses of Synbiotic

C- = negative control (without synbiotic application); C+ = positive control (commercial synbiotic); P = probiotic 2.5 x 10^5 cfu/ml and prebiotic 1.5 ppm; Q = probiotic 5.0 x 10^5 cfu/ml and prebiotic 1 ppm; R = probiotic 7.5 x 10^5 cfu/ml and prebiotic 0.5 ppm; S = probiotic 10^6 cfu/ml and prebiotic 2 ppm

treatment S were higher than in treatment C+. This is presumably because in the application of treatment S was growth the *Nutrient Broth* media while in treatment C+ the application was dilute by water media so that nutrients for bacteria to grow in the rearing tank were more contained in the rearing tank of treatment S. However, the treatment S is not significantly different with treatment C+ (11.1 log cfu/ml and 11 log cfu/ml) (P>0.05).

3.5 Effects of Synbiotic Application to Total *Vibrio* sp. Bacteria in the Rearing Water

The water used in this research was sea water that has been sterilized with 150 ppm chlorine so that *Vibrio* sp. in the water of rearing tanks in all treatments are <1 x 10^2 cfu/ml. While the results of the total of *Vibrio* sp. at the end of the rearing period shows that by applying synbiotics that is the mixture of yam paste as prebiotics and *Bacillus* sp., *Lactobacillus* sp., and Anoxygenic Photosynthetic Bacteria (APB) as probiotics can reduce the amount of *Vibrio* sp. in the water of rearing tanks (Figure 5). According to Triandini and Suryadi, who stated that the antibacterial produced by *Bacillus lentus* could inhibit Gram negative bacteria *(Proteus mirabilis)* [18]. According to the research by Sumardi *et al.* (2019b) which showed that *Bacillus* isolated with Anoxygenic Photosynthetic Bacteria (APB) could reduce the growth of *Vibrio* sp.. Anoxygenic Photosynthetic Bacteria (APB) had a longer lifetime and *Bacillus* had anti-microbial substances which could inhibit the growth of *Vibrio* sp. [19]. Furthermore, *Lactobacillus* sp. used is also known to inhibit the growth of *Vibrio* sp. It was previously reported by Jannah et *al.*, (2018) *Lactobacillus* sp. is a lactic acid bacteria (LAB) which produces lactic acid and bacteriocin that can inhibit the growth of the pathogen and provides acidic pH in the tract of digestive [14].

Meanwhile, prebiotics can act as a source of nutrition for probiotic bacteria so that they can maximize the ability of probiotic bacteria to inhibit the growth of *Vibrio* sp. on maintenance water. So that the application of probiotics (*Bacillus* sp., *Lactobacillus* sp., and APB) and prebiotics (oligosaccharide extract from yam paste) as synbiotics can inhibit the growth of *Vibrio* sp.

3.6 Effects of Synbiotic Application to Water Quality in the Rearing Water

Water quality parameters are temperature, pH, salinity, and ammonia (NH3) levels. Water quality reference based on SNI 7311: 2009 [20]. The results of

measurements of temperature, pH, salinity, and ammonia are presented in Table 2. During the rearing period, the temperature in each treatment had no differences in the morning, afternoon, and night. So that temperature has no effect to the survival and growth rates in each treatment. Whereas, The treatment P, Q, R, and S are not significantly different from treatment C- and C+ as negative control and positive control. This proves that the application of synbiotics does not affect changes in rearing water pH and changes in the survival rate and growth of shrimp larvae not affected by pH. While the results of salinity measurement, in treatments P, Q, R, and S have no significant differences with C- (negative control) and C+ (positive control). Thus explaining that application of synbiotics does not affect changes in rearing water salinity.

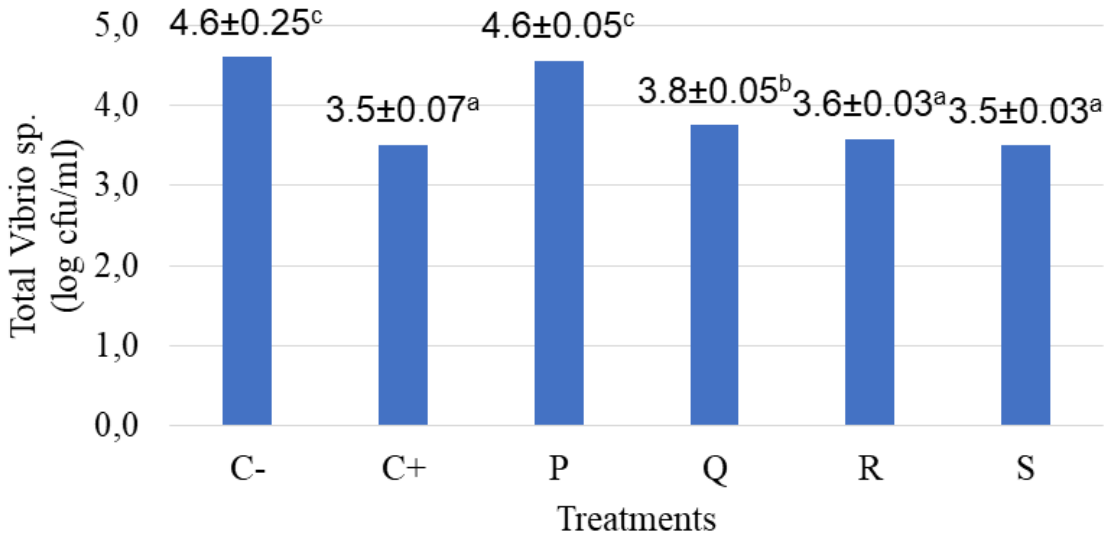

Figure 5. Total *Vibrio* Sp. in the Water of Rearing Plastic Tanks with Different Doses of Synbiotic

C- = negative control (without synbiotic application); C+ = positive control (commercial synbiotic); P = probiotic 2.5 x 10^5 cfu/ml and prebiotic 1.5 ppm; Q = probiotic 5.0 x 10^5 cfu/ml and prebiotic 1 ppm; R = probiotic 7.5 x 10^5 cfu/ml and prebiotic 0.5 ppm; S = probiotic 10^6 cfu/ml and prebiotic 2 ppm

Table 2. Measurement of Temperature, Ph, Salinity and Ammonia in the Water of Rearing Plastic Tanks With Different Doses of Synbiotic

Parameter		C-	C+	P	Q	R	S	SNI 7311:2009
Temperature (°C)	Morning	29.0	29.0	29.0	29.0	29.0	29.0	29-32
	Afternoon	30.0 – 31.0	30.0 – 31.0	30.0 – 31.0	30.0 – 31.0	30.0 – 31.0	30.0 – 31.0	
	Night	29.0 – 30.0	29.0 – 30.0	29.0 – 30.0	29.0 – 30.0	29.0 – 30.0	29.0 – 30.0	
pH (unit)	-	8.1 – 8.3	8.0 – 8.3	7.9 – 8.3	7.8 – 8.3	7.8 – 8.3	7.8 – 8.3	7.5 – 8.5
Salinity (ppt)	-	29.0 – 31.5	29.5 – 32.0	29.5 - 32,0	29.5 – 32.0	29.5 – 32.0	29.3 – 32.0	29-34
Ammonia (NH_3)	Zoea-1	0.0073	0.0073	0.0073	0.0073	0.0073	0.0073	< 0,1
	PL-1	0.0074	0.0071	0.0072	0.0061	0.0050	0.0046	
	PL-8	0.0075	0.0027	0.0068	0.0052	0.0047	0.0038	

A = negative control (without synbiotic application); B = positive control (commercial synbiotic); C = probiotic 2.5 x 10^5 cfu/ml and prebiotic 1.5 ppm; D = probiotic 5.0 x 10^5 cfu/ml and prebiotic 1 ppm; E = probiotic 7.5 x 10^5 cfu/ml and prebiotic 0.5 ppm; F = probiotic 10^6 cfu/ml and prebiotic 2 ppm

The concentration of ammonia (NH3) was checked three times during the rearing period when shrimp larvae in the zoea-1 stage before the application of synbiotic treatments, when shrimp larvae in the post-larvae-1 stage, and when shrimp larvae in the post-larvae-8 before harvesting. The results of ammonia concentration during the rearing period presented in Table 2. The treatment C+, P, Q, R, and S decreased the concentration of ammonia from zoea-1 stage, post-larvae-1 stage, and post larvae-8 stage. However in treatment C- (negative control) increased the concentration of ammonia. Differences in the decrease of concentration of ammonia from lowest to highest are in the treatment of P, Q, R. S, and C+ as the positive control. This is proving that the application of synbiotics can reduce the concentration of ammonia in rearing water.

Ammonium nitrogen can be transformed into nitrate nitrogen by the combination of nitrite oxidizing bacteria and ammonia-oxidizing bacteria [24]. Oligosaccharides that contained in yam flour as a prebiotic can be the source of nutrition for Bacillus sp. IBK3, *Bacillus* sp. UJ131, *Lactobacillus* sp., and APB as probiotics in rearing water or in the digestion of shrimp larvae, which can degrade ammonia. As in Solikhin's research which explained that the Bacillus sp. could degrade ammonia [21]. According to Narmatha et al., (2017) *Lactobacillus fermentum* as a probiotic in *Macrobrachium rosenbergii* culture, it could reduce ammonia levels [22]. Likewise, Anoxygenic Photosynthetic Bacteria (APB) were potential candidates for bioremediation agents and could reduce the ammonia compounds by 62% [23].

The water quality parameters are in the normal range following SNI 7311: 2009 [20]. Then it can be concluded that the change in the survival rate and growth of absolute length in the white shrimp larvae in the treatment is not caused by the rearing water quality.

4. Conclusion

This study concluded that the best synbiotic formula is S treatment or the addition of synbiotics which is a mixture of 10^6 cfu/ml probiotics (*Bacillus* sp. IBK3, *Bacillus* sp. UJ131, *Lactobacillus* sp., and Anoxygenic Photosynthetic Bacteria) and 2 ppm prebiotics (oligosaccharide extract from yam flour). The formula increased the survival rate up to 87.7%, with the total density of bacteria and *Vibrio* sp. in sequence 11.1 log cfu/ ml and 3.5 log cfu/ml. Whereas the best results of absolute length growth are shown in P treatment or the addition of synbiotics which were a mixture of 2.5 x 10^5 cfu/ml probiotics (*Bacillus* sp. IBK3, *Bacillus* sp. UJ131, *Lactobacillus* sp., and Anoxygenic Photosynthetic Bacteria) and 1.5 ppm prebiotics (oligosaccharide extract from yam flour) which is 3.8 mm.

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