Study of *Bacillus methylotrophicus* as a Probiotic Candidate Bacteria With Different Concentration Against *Aeromonas hydrophila* on Water as a Cultivation Media of Tilapia (*Oreochromis niloticus*)

Study of *Bacillus methylotrophicus* as a Probiotic Candidate Bacteria With Different Concentration Against *Aeromonas hydrophila* on Water as a Cultivation Media of Tilapia (*Oreochromis niloticus*)

Putri Agustina, Sarjito, Alfabetian Harjuno Condro Haditomo*

Aquaculture Departement, Diponegoro University, Semarang, Indonesia
Jl. Prof. Soedharto, S.H Tembalang, Semarang, Indonesia. 50275
Corresponding author: condrohaditomo@gmail.com

Abstract. *Aeromonas hydrophila* is still the major problem of the decline in tilapia production. The use of probiotics as disease prevention agents is one of alternative strategy. One of probiotic candidate that has been molecularly identified through 16s RNA was *Bacillus methylotrophicus* that was known as a bacteria that produces cyclic lipopeptides belonging to surfactin, iturin, and fengycin families. The aim of this research was to study *B.methylotrophicus* in inhibiting *A.hydrophila* in *Oreochromis niloticus* culture. This research consisted of in vitro and in vivo test that used experiment method with completely randomized design, 4 treatments (density of 1 fish/l) and 3 replications. The treatments were various mixture concentration of *A.hydrophila* at 10^2 CFU ml^{-1} with bacterial *B.methylotrophicus* (a) 0, (b) 10^7, (c) 10^8, (d) 10^9 CFU ml^{-1}. Experimental animals used 120 fish at average weight of 13,8±1,6 g. Based on the *in vitro* test, *B.methylotrophicus* able to inhibit *A.hydrophila* categorized as bactericidal antibiotic and the most powerful concentration was 10^9 CFU ml^{-1} with clear zone of 24,9±4,2 mm. *In vivo* test did not significantly effect on survival rate, could slow down the growth of *A. hydrophila*, and had effect on dynamics of leukocytes, erythrocytes, hemoglobin, hematocrit value. Treatment D showed the highest survival rate (56.7%), followed by C (50,00%), B (43,33%), and A (30,00%). The value of leukocytes, erythrocytes, hemoglobin, hematocrit were 66.300–208.700 cells/mm^3, 0,3–2,17x10^6 cells/mm^3, 21,8-32,7%, 3,1-7,4 g/dl, respectively. The total bacteria viable count (TBC) in the water and *A.hydrophila* during treatment were 1,66x10^{11}–1,25x10^{17} CFU ml^{-1} and 3,3x10^4–1,7x10^7 CFU ml^{-1}. This results showed that *B.methylotrophicus* could inhibit growth of *A.hydrophila* in in vitro, and able to increase the SR until 26,7% in in vivo test.

1. Introduction

Tilapia (*Oreochromis niloticus*) is a second most farmed fish that is widely cultivated throughout the world and its production has quadrupled in the past decade because it is easy to cultivate, high demand and stable market prices [1]. Intensification of tilapia aquaculture faced various obstacles including fish disease problems that have an impact on economic losses. One type of fish disease that is often found in tilapia and freshwater fish in general around the world is bacterial disease caused by *Aeromonas hydrophila* [2].

A.hydrophila is unicellular, Gram negative, motile, and opportunistic heterotrophic bacteria that can cause death in fish in a short time. This is made possible by the presence of extrasellular toxin

products such as haemolysin, aerolysin, cytolysin, enterotoxin, amylase, and other toxins released. *A. hydrophila* is one of agent that causes Motile Aeromonas Septicemia (MAS) which has signs of fish with stomach edema, inflammation around the wound, bleeding in the fish's body, rotting gills, ulcers, weakness and prominent eyes (exophtalmia) [3].

Various efforts have been made to overcome the infection of *A.hydrophila*, one way by using antibiotics. The use of antibiotics have some disadvantages. Firstly, pathogens become resistant and the other, residues arise in fish that will be dangerous if consumed by humans. Another safer solution was the use of various plant extracts and the use of probiotic bacteria. Overall, further research is still needed to solve the problems [4].

There are several probiotic bacteria that have been shown to inhibit the growth of pathogenic *A.hydrophila*, namely *Bacillus subtilis*, *L. plantarum* and *B.megaterium*, *Bacillus* sp., *Lactobacillus* sp. and *Arthrobacter* sp., *Tetraselmis suecica* [2,5,6,13], with treatment via the feed or to the cultivation water in aquaculture [1]. Other probiotic candidate bacteria that have the potential to inhibit the growth of *A.hydrophila* was *Bacillus methylotrophicus* bacteria [6], with CBL20 isolate code. *B.methylotrophicus* is able to actively inhibit pathogenic bacteria both Gram positive and Gram negative [7]. Its content of cyclic lipopeptides included in the surfactin and iturin groups is thought to be active substances that inhibit the growth of pathogenic bacteria [8], including pathogenic bacteria in humans [9]. The potential of *B.methylotrophicus* has not been applied to fish pathogenic bacteria, including *A.hydrophila* in tilapia fish.

The aim of this study were to examine the ability of *B. methylotrophicus* to inhibit *A. hydrophila* in cultivation media, survival rate level, blood profile, concentration of bacteria in water and fish clinical signs. This research was conducted from January to April 2018 at Laboratory of the Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang.

2. Research Methods

2.1. Experimental materials and animals

The preparation phase consisted the sterilization of tools and materials, acclimatization of tilapia fish, the preparation of bacterial media agar, bacterial culture and enhancement of bacterial virulence. Microbiological tools sterilization based on previous studies [32]. The agar media used was Tryptic Soy Agar and Tryptic Soy Broth media (TSA, TSB - Merck) for the in vitro test [10] and pure isolate culture. Selective media of Glutamate Starch Phenol (GSP) as agar medium for *A.hydrophila*.

The experimental fish were tilapia (*O. niloticus*) originating from the tilapia`s hatchery Mina Muncul (Ambarawa, Semarang) with stocking density of 1 fish/L, 10 fish/aquarium, a total of 120 fish with an average weight of 13.8±1.6 g. Artificial feed (pellets) were given using *at satiation* method 2 times/day (08.00 a.m and 16.00 p.m) [11,32].

The probiotic candidate bacteria was obtained by Haditomo *et.al.* [6] with CBL20 isolate code which was the result of screening from tilapia culture mud bacteria in Boyolali, Central Java. This bacteria has been identified by 16sRNA sequence analysis and then compared with sequences at NCBI so that the type of bacterial *B.methylotrophicus* strain XJAJ2 was obtained with 100% homology level. Pathogenic bacteria was *Aeromonas hydrophila*. The pure isolates of *A. hydrophila* were obtained from the bacterial isolation process in tilapia cultivation media in the Wet Laboratory of Diponegoro University which had been conducted by the series of biochemical tests for the identification.

2.2. Experimental setup

The freshwater cultivation media were precipitated in a reservoir for a week and has been sterilized using 10 ppm of liquid chlorine and 20 ppm of sodium thiosulfate. 12 aquariums were used with a size of 40 cm x 30 cm x 40 cm and water volume was 10 L/aquarium, equipped with an aeration system.

The enhancement of bacterial virulence started by culturing *A.hydrophila* bacteria in TSB liquid media, and incubated for 24 hours. Tilapias were infected with *A. hydrophila* isolates. The bacterial infection process was carried out by injecting Intramuscularly 3 times [12]. Bacteria were cultured on

GSP media. The results showed yellow colonies then it was purified on TSA media. Some biochemical test has been done to ensure that was *Aeromonas* sp. Pure isolates were then cultured in TSB media for retesting until they were ready for the in vivo test.

The in vitro test carried out was the inhibition zone test was done by visual observation and measuring its diameter. It used paper discs which previously have been soaked in *B.methylotrophicus* in different concentration. The disc papers were put on TSA media that have been spread with *A.hydrophila* then waiting for 24 hours [38].

Preliminary tests were carried out through the application of probiotic candidate bacteria in tilapia cultivation media which contained pathogenic bacteria *A.hydrophila* 10^4 CFU ml^{-1} and observed the response of tilapia for 14 days of maintenance. The *B.methylotrophicus* concentration tested was 10^3, 10^4, 10^5 CFU ml^{-1} consisted of 4 treatments (with 1 control treatments) and 3 times repetition. Each replication consisted of 20 fish (density of 2 fish/l). The parameters observed were survival rate, clinical symptoms, and the amount of bacterial density in the cultivation medium [3,6,38].

The in vivo test [6], using 10 L of sterile culture media (water) was prepared. Then the medium was given *A.hydrophila* 10^2 CFU ml^{-1} and *B.methylotrophicus* with a density of 0, 10^7, 10^8, 10^9 CFU ml^{-1}. 10 experimental fish in each aquarium (stocking density of 1 fish/l) were put into the aquarium which was given bacteria and observed for 14 days. During the treatment there was no water change. The management of water quality was carried out by measuring the water quality variables during the study such as temperature (^{0}C), pH, and dissolved oxygen (DO) used water quality checker, pH meter, and alcohol thermometer.

The method used was experimental with a completely randomized design (RAL) of 4 treatments and 3 replications. The treatments consisted of:

Treatment A (density of *A.hydrophila* 10^2 CFU ml^{-1} + *B.methylotrophicus* 0 CFU ml^{-1});
Treatment B (density of *A.hydrophila* 10^2 CFU ml^{-1} + *B.methylotrophicus* 10^7 CFU ml^{-1});
Treatment C (density of *A.hydrophila* 10^2 CFU ml^{-1}+ *B.methylotrophicus* 10^8 CFU ml^{-1}) and
Treatment D (density of *A.hydrophila* 10^2 CFU ml^{-1} + *B.methylotrophicus* 10^9 CFU ml^{-1}).

2.3. Data collection

2.3.1. Survival rate. Tilapia's survival rate according to Effendi [14] can be calculated using the formula:

$$SR = \frac{Nt}{No} \times 100\% \quad (1)$$

Note: SR = Survival rate (%), Nt = Number of tilapia that lives at the end of observation (ind), No = Number of tilapia from beginning (ind)

2.3.2. Abundance of bacteria in water. Calculation of total bacteria in water was carried out at the beginning (day 1), middle (7), and end of the study (14). Total bacteria in water were calculated using the total plate count method [15]. The media used was TSA media to calculate the total viable bacterial count (TBC), GSP for the total *A. hydrophila*.

2.3.3. Clinical symptoms. Clinical symptoms observed visually for 14 days such as behavior and fish morphological changes [12].

2.3.4. *Blood profiles.* Blood samples were taken on 0, 1, 5, 9, and 13 days. Blood sampling used a 1 ml syringe directed to the caudal artery with a slope of 45^0. Injection syringes were first washed with anti-coagulant (EDTA). The blood taken was inserted in the microtube. Blood profiles observed in this study were total erythrocytes, hematocrit levels, total leukocytes, and hemoglobin levels. The total erythrocyte and leukocyte calculation method refered to Blaxhall and Daisley [16] using Turk's reagent to observe leukocytes and Hayem reagent for observing erythrocytes. Calculation of erythrocytes and leukocytes used the formula:

Total erythrocytes = number of calculated cells x 10^4 cells/mm^3;
Total leukocytes = Number of calculated cells x 50 cells/mm^3.

The procedure for calculating hemoglobin levels refered to the Sahli method and expressed in unit of g/dl. Hematocrit levels were measured according to Anderson and Siwicki [17], the length of the blood that precipitated (a) and the total length of the volume of blood contained in the tube (b) was measured using a ruler. Hematocrit levels are expressed as percent (%) of the volume of blood cell solids. Hematocrit calculation formula was:

$$\text{hematocrit level (\%)} = \frac{a}{b} x\ 1 \quad (2)$$

2.3.5. Water quality. Water quality parameters included dissolved oxygen, pH, and temperature. During cultivation, the water was not siphoned and water replacement was not carried out [10]. Water quality measurements were carried out 3 times in 14 days of treatment, that were at the beginning (day 1), middle (7), and the end of the study (14) [18]

2.3.6. Data analysis. The inhibitory zone, survival rates, blood profile, and clinical symptoms were used descriptive analysis [10].

3. Results

3.1. In vitro test

Based on the inhibition zone test, the results were presented in Table 1.

Table 1. Diameter of Inhibition Zone by Serial Concentration of *B. methylothropicus* by In Vitro Test.

No.	*B.methylotrophicus* concentration (CFU ml^{-1})	Diameter of inhibiton zone (mm)			Average±SD (mm)
		1	2	3	
1	0	0	0	0	0
2	10^1	0	0	0	0
3	10^2	0	0	0	0
4	10^3	7	7,2	7	7,07±0,12
5	10^4	8	8	7,2	7,73±0,46
6	10^5	9	7	8	8±1
7	10^6	8,3	8,6	8,6	8,5±0,2
8	10^7	12,8	14,7	18	15,2±2,63
9	10^8	10	7	7	8±1,7
10	10^9	20,6	29	25	24,9±4,2

The highest value showed the density of *B.methylotrophicus* 10^9 CFU ml^{-1} was 24.9 ± 4.2 mm. This value included a very strong inhibitory zone and categorized as bactericidal antibiotic. The density of *B.methylotrophicus* then used in the in vivo test was 10^7, 10^8, and 10^9 CFU ml^{-1}.

3.2. Survival rate

The highest survival rate is in treatment D (56.67%) followed by C (50.00%), B (43.33%) and the lowest was treatment A (30.00%). The addition of *B. methylotrophicus* could increase the survival rate until 26.67%. These survival rate showed different mortality pattern during treatment. The fish mortality pattern showed in the Figure 1.

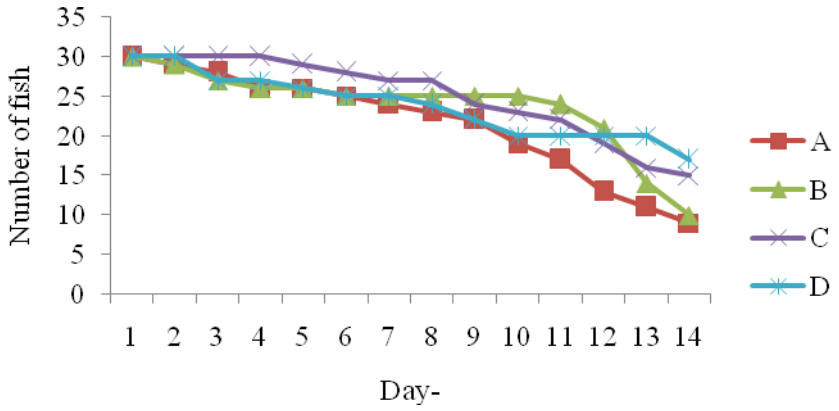

Figure 1. Fish Mortality During Treatment *B. methylothropicus* against *A. hydrophila*
A) density of *B.methylotrophicus* 0 CFU ml^{-1}; B) 10^7 CFU ml^{-1}; C) 10^8 CFU ml^{-1}; D) 10^9 CFU ml^{-1}

Tilapia mortality patterns showed that fish mortality occurred on day 2 in treatment A and B, while treatment D death began on day 3, and the fish death of treatment C occurred on day 5. Death increased after 9th day observations for all treatments.

3.3. Abundance of bacteria

The total bacteria in the cultivation media and *A.hydrophila* increased from the beginning to the 14th day, only in treatment D showed a decrease in the number of bacteria at the end of the study. The total bacteria viable count (TBC) in the water and *A.hydrophila* during treatment were $1{,}66x10^{11}$–$1{,}25x10^{17}$ CFU ml^{-1} and $3{,}3x10^{4}$–$1{,}7x10^{7}$ CFU ml^{-1}. Treatment of A and B has increased from the beginning of the study until the end of the study. The total bacterial end of the study in treatment A was 1.25×10^{17} CFU ml^{-1}, there were 1.69×10^{7} CFU ml^{-1} of *A.hydrophila* bacteria, the ratio of *A.hydrophila* to the whole bacteria was 1: 10^{10}. Treatment C showed a decrease in the number of bacteria in the middle of the study (day 7) to $4.9x10^{12}$ CFU ml^{-1} with the amount of *A.hydrophila* $2x10^{5}$ CFU ml^{-1}, then increased at the end of the study. The D treatment showed an increase in the number of bacteria on the 7th day of the study and decreased at the end of the study to $5.1x10^{12}$ CFU ml^{-1} with the amount of *A.hydrophila* $5.5x10^{6}$ CFU ml^{-1}. Comparison of the number of *A.hydrophila* with overall bacteria on day 1 is 1: 10^7 for treatment A, B while the treatment C, D was 1: 10^9. Day 7 is 1: 10^9 for treatment A, B while treatment C is 1: 10^7 and treatment D is 1: 10^{10}. Day 14 shows a value of 1: 10^{10} for treatment A, B, C while treatment D is 1: 10^6.

3.4. Clinical symptoms

Clinical symptoms of tilapia for 14 treatment days were presented in Table 2.

Table 2. Clinical Symptoms of Behaviour and Morphology Changes of Tilapia

Day-	Treatment A			Treatment B			Treatment C			Treatment D		
	1	2	3	1	2	3	1	2	3	1	2	3
1	a,c	a,c	a,c	a, c	a	a	a	a	a	a	a	a
2	b,c	a,c	b,c	a,c	a	a	a	a	a	a	a	a
3	b,c,d	a,c	b,c	b,c	a	a,c	a	a	a,c	a	a	a,c
4	b,c,d	a,c	a,c	b,c	a	a,c	a,c,d	a,e	a,c	a	a	a,c
5	b,c,d	a,c	a,c	b,c	a,c	a,c	a,c,d	a,e	a,c	a	a	a,c
6	b,c,d	a,b	b,c	b,c	a,c	a,c	a,c	a,c,e	a,c	a,d	a	a,c
7	b,c,d	b,c,e	b,c,d	b,c	b,c	a,c	a,c	b,c,e	a,c	a,d	a	a,c
8	b,c,d	b,c,e	a,c,d	b,c	a,c	b,c	b,c	b,c,e	a,c	a,c	a	a,c
9	b,c,d	b,c,e	a,c,d	b,c	b,c	b,c,d	b,c	b,c	b,c	a,c	a	a,c

10	b,c,d	b,c,e	b,c,d	b,c,d	b,c	b,c	b,c	b,c	b,c	b,c	a	b,c
11	b,c,d	b,c,e	b,c	b,c,d	b,c	b,c	b,c	b,c	b,c	b,c	b,c	b,c
12	b,c,d	-,c,e	b,c	b,c,d	b,c	b,c	b,c	b,c	b,c	b,c	b,c	-,c
13	b,c,d	-,c,e	-,c	b,c,d	b,c	b,c	b,c	b,c	b,c	b,c,d	b,c	-,c
14	b,c,d	-,c,e	-,c	b,c,d	b,c	b,c	b,c	b,c	b,c	b,c,d	b,c	-,c

-: fish did not respond to feed, and very passive, a: active, fast feed response, b: passive, swimming unstable and near aeration, slow feed response, c: excess mucus, pale skin colour, stretched dorsal fin, d: fin thin tail, loose scales, e: protruding eyes (exopthalmia)

Clinical symptoms of tilapia in treatments A, B, C, D presented in Table 2 indicated changes in behavior and morphology. Clinical symptoms in treatment A showed passive responses, swimming unstable and near aeration, slow feed response, excess mucus, pale skin color, stretched dorsal fin, thin caudal fin, peeling scales (Figures 2a, 2b, 2c) . Some fish also showed wound or ulcers on the surface of the body, and prominent eyes (exopthalmia). Three days near the end of the study, tilapia showed that changes in clinical symptoms that fish did not respond to feed.

Figure 2. Clinical Symptoms of Tilapia During Treatment
a) Thin fins; b) Scales peeling; c) Pale fish color

Tilapia showed clinical symptoms like thin fins on day 4 of treatment A, while treatment D occurred on day 6. Day 7,8,9 tilapia showed more changes in morphology and behavior in treatment A, passive, stretched dorsal fin, thinned fins, and some exopthalmia. The clinical symptoms of treatment B were passive, excess mucus, stretched fins. Treatment C showed the presence of exopthalmia fish, while the treatment D was still active, but the body paled and fins stretched.

Days 10 and 11 did not show changes from the previous day in treatments A, B and C, while treatment D showed changes in morphology and behavior in the form of passivity, slow feed response, swimming near aeration, excess mucus, pale color and dorsal fin stretch. Then treatment A tilapia fish on days 12, 13 and 14 showed changes in clinical symptoms such as fish did not respond to feed, slow motion and very passive. Treatment of B and C did not experience clinical symptom changes from the previous day, whereas in treatment D showed excessive mucus, pale, and passive, and did not respond to feed.

3.5. Blood profile

The results of blood profile measurements (erythrocytes, leukocytes, haemoglobin, haematocrit) on days 0, 1, 5, 9 were presented in Table 3.

Table 3. Results of Blood Profile Measurements During the Study

Treatment	Day	Erythrocytes (cells/mm^3)	Leukocytes (cells/mm^3)	Haemoglobin (g/dl)	Haematocrit (%)
A	0	2.46±0.38	1.93±0.92	7.37±1.46	32.20±1.91
	1	1.94±0.54	3.36±2.51	5.47±2.50	30.27±1.62
	5	1.20±1.05	4.65 ± 3.85	5.10±1.15	27.23±13.45
	9	0.64±0.44	8.54 ± 2.29	4.07±0.12	25.37±0.64
	13	0.98±0.49	7.42±0.75	4.73±0.42	27.07±3.83

B	0	2.46±0.77	2.12±0.37	6.07±0.90	30.67±1.15
	1	2.23±0.29	4.12±2.42	5.40±0.61	28.07±0.81
	5	2.14±1.64	4.35±2.29	5.20±1.20	26.00±3.61
	9	1.64±0.52	5.81±4.31	4.73±0.31	25.67±1.15
	13	2.21±0.20	4.91±0.37	5.00±0.20	30.47±0.12
C	0	2.26±0.24	2.20±0.36	6.47±0.55	32.00±2.00
	1	1.96±1.09	5.43±1.52	6.03±1.36	30.07±3.29
	5	2.23±1.99	4.80±2.25	6.33±0.15	30.20±0.35
	9	2.33±0.07	5.25±1.78	6.47±0.46	31.53±2.16
	13	2.41±1.01	5.63±3.46	6.77±0.42	33.47±3.23
D	0	2.43±0.14	2.76±0.33	5.87±1.99	30.60±1.51
	1	1.92±0.59	5.11±0.34	5.43±0.59	30.07±0.46
	5	2.38±1.61	6.80±2.41	6.47±0.55	31.50±5.89
	9	2.46±0.55	6.60±0.94	6.63±0.55	32.13±1.60
	13	2.63±0.11	5.48±2.24	6.90±0.35	32.80±1.91

The average leukocytes value of tilapia increased from day 0 to day 9 and decreased on day 13, except in treatment C which decreased on day 5. The highest average leukocyte value on day 13 was in treatment A (74,000 cells/mm^3). The average value of leukocytes is still in the standard value of leukocytes in tilapia, except in day-13. Fish experienced a decrease in total mean erythrocytes from day 0 to day 13. This value is still included in the category of normal teleostei fish erythrocyte levels, except in day-13. The pattern of erythrocyte increase and decrease was the same as the pattern in hematocrit and hemoglobin levels. Healthy fish hematocrit levels (day 0) averaged between 30.7-32.2%. Healthy fish hemoglobin levels (day 0) averaged between 5.9-7.4 g/dl.

3.6. Water quality

Measurement of water quality variables was carried out at the beginning, middle, and end of the study. The results of measurements during the study on all treatments are presented in Table 4.

Table 4. Range of Water Quality Parameters During Experiment

Parameters	Treatments				Optimum Range
	A	B	C	D	
DO (mg/l)	1,64 – 3	1,87 – 3,1	1,86 – 3,1	1,88 – 3,0	≥3[a)]
Suhu (^oC)	26 – 27	27– 28	26 – 28	26 – 28	25 – 32[a)]
pH	6 – 8	6 – 8	6 – 8	7 – 8	6,5 – 8,5[a)]

[a)] SNI (2009)

Water quality were not in the optimum range but it still suitable for use in tilapia fish rearing media. Even so, oxygen levels and low pH occur at the end of the study indicating that fish mortality at the beginning and mid-treatment is not caused by improper water quality. During the study there was no water changes.

4. Discussion

B.methylotrophicus bacteria in in vitro test could inhibit the growth of *A. hydrophila* bacteria. This is evidenced by the inhibition zone (clear zone) produced on disc paper (Figure 1). The ability of inhibition of *B.methylotrophicus* to *A. hydrophila* was very strong at a density of 10^9 CFU ml^{-1}, strong for the density of 10^7 CFU ml^{-1}, and moderate for the density of 10^8 and 10^6 CFU ml^{-1}. The inhibition area with a value of more than 20 mm was very strong in inhibiting bacterial growth. The inhibition area of 10 mm-20 mm is relatively strong and the area of inhibition 5-10 mm is classified as moderate and below 5 mm was classified as weak in inhibiting bacterial growth [19,38]. The formation of a large inhibitory zone at a density of *B. methylotrophicus* 10^9 CFU ml^{-1} showed an antagonistic

interaction between probiotic candidate bacteria and *A.hydrophila*. This interaction can be related because of the nutritional competition of bacterial growth media and the presence of antimicrobial or bacteriocin compounds produced by *B.methylotrophicus*. The competition in nutrient absorption was a common phenomenon in natural habitats. Some bacterial species used antagonistic activity or inhibiting devices or organs as weapons against competitors [20]. Generally bacteria produced several types of anti-microbial compounds to inhibit or kill other competing bacterial species [21].

The ability of antibiotics in *B.methylotrophicus* was included in the bactericidal category because the mechanism of surfactin was to damage the bacterial cell wall so cause the death of pathogenic bacteria. It was proven by the presence of inhibitory zones after 48 hours of observation. *B.methylotrophicus* was able to inhibit *A.hydrophila* because it contained a wide range of cyclic lipopeptides. Polypeptides or lipopeptides are polymers composed of several peptides which are bound to carboxyl (COOH) groups with amino groups. One or more polypeptides can form proteins, for example enzymes. Polypeptides are formed through the process of gene expression that occurs in cells. According to [22], Iturin consists of seven amino acid residues that are hydrophilic and hydrocarbon tails with 10-13 carbons in length which are hydrophobic. Iturin and surfactin are examples of antibiotics that can inhibit fungal growth [23]. Furthermore [8], explained the structural character and identification of cyclic lipopeptides produced by *B. methylotrophicus* DCS1. Rude lipopeptide samples from *B.methylotrophicus* were composed of about 40% protein. Various amino acids were detected in DCS1 (*B.methylotrophicus*) rude lipopeptides, most of the amino acids that make up the composition of lipopeptides belong to the surfactin, iturin and fengycin families. Surfactin is an antibiotic that has work as a biosurfactant, surfactin can damage the permeability of cell membranes by decreasing surface tension [23]. Surfactin which is a cyclic lipopeptide in addition to lowering the surface tension of a liquid also damages spheroplast and other bacterial protoplast near the producing bacteria. The function of surfactin as an antimicrobial is related to its ability to bind hydrophobic molecules to bacterial membranes [24]. The effectiveness of surfactin as an antimicrobial compound depends on the surfactin concentration and the resistance of the surrounding microorganism membrane which can be inhibited [25].

Survival rate from lowest to highest at the end of the study was treatment A (concentration of *B. methylotrophicus* 0 CFU ml^{-1}) which was 30.00%, followed by treatment B (concentration of *B. methylotrophicus* 10^7 CFU ml^{-1}) which was 43.33%; C (concentration of *B.methylotrophicus* 10^8 CFU ml^{-1}) is 50.00%; D (concentration of *B.methylotrophicus* 10^9 CFU ml^{-1}) 56.67%. It showed that *B.methylotrophicus* could increase survival rate until 26,67%. It caused the presence of surfactin and iturin and showed that the effectiveness of probiotics is influenced by bacterial composition, dose/concentration used, age and quality of probiotics [13].

Tilapia mortality patterns showed varying values. Day 1, there were no deaths in all treatments. Clinical symptoms of tilapia showed pale fish, excess mucus, dorsal fins stretched but swimming was still normal and active in all treatments. This clinical symptom showed stress response from fish due to 10^4 CFU ml^{-1} of *A.hydrophila* bacteria in all treatments with total bacteria in water was 10^{11} CFU ml^{-1} for treatment A and B, while treatment C and D was 10^{13} CFU ml^{-1}. The comparison of *A.hydrophila* bacteria with total bacteria was 1: 10^7 for treatment A, B, and 1: 10^9 in treatment C, D. Bacterial density on the first day has not caused the death of fish but has been able to increase the physiological response of fish to the presence of infection as evidenced by the increase in total leukocytes, decreased erythrocyte values, hematocrit and hemoglobin of tilapia all treatments compared to healthy tilapia. This reduction was still within normal limits. Lower hematocrit and hemoglobin values indicated a hematological reaction of tilapia. Hematocrit values below 30% indicated erythrocyte deficiency. Infectious diseases in fish and decreased appetite can cause hematocrit values to decrease [26].

On the second day of observation, 1 tilapia died in treatment A and B. Clinical symptoms of tilapia in treatment A and B fish were passive, feed response is slow, but also there were fish with normal and active swimming conditions. Physiological conditions of fish treatment A and C showed a stress response due to the presence of *A.hydrophila* infection as evidenced by increased bacterial density and

total leukocytes, decreased erythrocyte values, hematocrit and hemoglobin. On the contrary, in treatment B and D there was a decrease in the number of leukocytes, an increase in the number of erythrocytes, hemoglobin, hematocrit. Although blood values improved in treatment B, fish have not been able to recover from the infection that occurred from the first day. This showed that treatment A and B had *A.hydrophila* infection as evidenced by bacterial isolates growing yellow on GSP medium and leukocyte increasing. A total number of leukocytes often increases after infection indicating that leukocyte cells work as cells that phagocytes to bacteria so they cannot develop and spread virulence in the host's body [27].

Day 3 and 4 showed the death of 1-2 fish in A, B, and D. Treatment of C even though there was no death, still showed an infection response. The comparison between *A.hydrophila* 10^4 CFU ml^{-1} with total bacteria in water was 1: 10^7 - 1: 10^9 and this value capable of causing death. Clinical symptoms of tilapia indicated the presence of thin fins, loose scales, pale fish color, and excess mucus. Treatment C contained exopthalmia, but in treatment D tilapia still swim normally, pale and excess mucus. The blood profile still showed the same trend as the previous day. Clinical symptoms of protruding eyes were thought to be due to toxic caused by *A. hydrophila*, hemolysin toxin. It exotoxins (one of them hemolysin) that enter the body of the fish can spread through the blood and damage the choroid part of the eye, causing the eyes to experience hyperemia [28]. The presence of hyperemia in the choroid part causes exophthalmia. The working system of hemolysin toxin with the target breaking down red blood cells, so that the cells come out of the blood vessels, giving rise to a reddish color on the surface of the skin. The occurrence of ulcers is caused by the high density of bacteria in these locations, which was excreted in the infection process was also higher in that part, while some others enter the body following the blood flow to the organ [29].

The death at fifth day occurred at C and D treatment. There were no deaths in treatment A and B. Clinical symptoms of tilapia showed the presence of thinned fins, loose scales, and exopthalmia in treatment C, but in treatment D the clinical symptoms were only pale, excessive mucus, and feed response was still fast. Treatment of B and D showed decreasing in leukocyte values, erythrocytes, hematocrit and increased hemoglobin. The treatment of D decreased significantly indicating that tilapia was able to improve physiological conditions within 5 days compared to other treatments. It is suspected that there was a decrease in leukocyte response due to the decreased number of *A.hydrophila* infections, as evidenced by clinical symptoms. Even so, the deaths that occurred in treatment D showed that there were fish that had not fully experienced physiological improvement due to the infection of *A.hydrophila* 10^4 cfu/ml in water. Likewise treatment A and C also showed stress response. Treatment B showed a physiological improvement of fish because erythrocytes, hemoglobin, hematocrit increased, and leukocytes decreased. Probiotic candidate bacteria were thought to be able to improve or increase the number of erythrocytes. Some species of Bacillus can increase the value of erythrocytes, hemoglobin and hematocrit in rainbow trout (*Oncorhynchus mykiss* Walbaum) [30].

On the 6^{th} day there were deaths of 1 fish in all treatments. Clinical symptoms of tilapia showed the same conditions as the previous day, and meant that tilapia still had infection from *A.hydrophila* indicated by leukocyte values increased in treatment B and D, while treatments A and C showed decreased leukocytes. However, based on clinical symptoms all treatments showed a stress response. Clinical symptoms of this behavior [31], fish infected with *A. hydrophila* bacteria will show behavior changes such as swimming on the surface and irregular, and tend to swim tilted, inflammation characterized by swelling and injury, portruding of the eyeball (expothalmia), necrosis on the surface of the body, red anus.

On the 7^{th} day there were 2 fish deaths in treatment A and 1 fish in C. The amount of leukocytes, erythrocytes, hematocrit and hemoglobin were the same range as the previous day. There was no dead fish in treatment B and D. Clinical symptoms of tilapia were passive, thin fins, loose scales, some exopthalmia fish. Clinical symptoms showed the presence of *A.hydrophila* infection as evidenced by the increasing number of *A.hydrophila* in water which was 10^6 CFU ml^{-1} in treatment A and 10^7 CFU ml^{-1} in treatment B with a ratio of *A.hydrophila* and total bacteria in water was $1:10^9$ in treatment A and B, while the C and D treatment of *A.hydrophila* was 10^5 cfu/ml with a ratio of $1:10^7$ and $1:10^{10}$.

The number of these bacteria can cause death in tilapia. In this study the amount of *A.hydrophila* can cause death was between 10^4-10^7 CFU ml^{-1}. The concentration of *A.hydrophila* in culture media that can cause Motile Aeromonad Septicemia (MAS) in cyprinid was around 10^7-10^8 CFU ml^{-1}[32].

On the 8th day, there were 1 fish deaths in treatment A and D. All treatments experienced *A.hydrophila* infection with passive clinical symptoms, thinning fins, loose scales, pale and exopthalmia. The number of bacteria was more or less the same as the previous day. Fish blood showed the same trend as the previous day which showed that the fish was not in normal or sick condition. Leukocytes function to cleanse the body from foreign attacks including invasion of pathogens through immune response systems and other responses [27]. The condition of the sick fish will produce a large number of leukocytes to phagocytize bacteria and synthesize antibodies, while the decrease in the number of leukocytes that occur after infection indicated bacterial phagocytic activity and antibody synthesis has decreased.

Day 9th tilapia died in treatment A, C, and D. Clinical symptoms were still the same as the previous day which showed that tilapia had *A.hydrophila* infection as evidenced by the number of leukocytes treated with B and D, although leukocytes A and C decreased, tilapia in the two treatments still showed clinical symptoms of stressed fish. The 10th day of death occurred in treatments A, C and D. Clinical symptoms of tilapia were thin fins, decreased feed response, pale color, exopthalmia. The number of bacteria in the same range as the previous day. Tilapia showed a hematological response that indicated infection. The number of leukocytes in all treatments showed an increasing trend. This showed that the fish cannot fully recover. Amount of erythrocytes, hematocrit, hemoglobin showed a downward trend. This decrease was thought to be due to the presence of extracellular products produced by *A. hydrophila* including aerolysin and hemolysin [33]. This product was related to the level of virulence of the bacteria. Aerolysin and hemolysin show hemolysis activity against erythrocytes in vitro [34] and in vivo [35]. When *A. hydrophila* enters the host's body, the bacteria will produce lechitinase, an enzyme that destroys various tissue cells and is mainly active in lysing erythrocyte cells [36].

Day 11,12,13, and the 14th death of tilapia in all treatments with a relatively high amount compared to deaths in the previous days. Clinical symptoms showed that the fish continues to experience stress as evidenced by thinning fins, loose scales, pale color, excessive mucus, passivity, no response to feed, some exopthalmia fish. The values of leukocytes, erythrocytes, hematocrit, hemoglobin strengthen the suspected infection at the end of the study. The number of leukocytes in all treatments increased to 16.2-20.86 x 10^4 cells/mm^3. The mean value of these leukocytes was not in the normal value of leukocytes in tilapia due to stress. A stressful conditions can affect physiological activity and hemoglobin levels in fish [37]. The physiological state of fish's blood depended on environmental conditions such as humidity, temperature, and pH [38].

The number of erythrocytes, hematocrit, and hemoglobin decreased at the end of the study. The erythrocyte, hematocrit and hemoglobin values at the end of the study were 0.53-0.77x10^6 cells/mm^3; 21.8-25.2%; 3.13-4.37 g/dl, respectively. All of these values were not in the normal range of tilapia blood. Decreased hemoglobin values and hematocrit were suspected because fish faced sick conditions. That measurement of hematocrit was done if there was a suspicion of a disease that disrupts red blood cells, either excessive or deficient [39]. If there was a decrease in suspected erythrocyte levels also decrease due to infection in the body of the fish, so that erythrocytes become damaged. Decreased erythrocyte levels will cause hematocrit and hemoglobin also decrease. Conversely, increased hematocrit levels indicate that blood was in good condition and able to bind oxygen well [26].

The final *A.hydrophila* density of the study without *B.methylotrophicus* (treatment A) was 10^7 CFU ml^{-1}, while the population of *A. hydrophila* in the treatment of *B.methylotrophicus* (B, C, D) was 10^6 cfu/ml. The total population growth of bacteria found in the cultivation media at the end of the study showed the number of 10^{17} CFU ml^{-1} in treatment A, then in treatment B and C was 10^{16} CFU ml^{-1}, whereas in treatment D showed the number 10^{12} CFU ml^{-1}. Comparison of *A.hydrophila* with total bacteria was 1: 10^{10} for treatment A, B, C, while treatment D was 1: 10^6. This value indicated the

lowest bacterial population in the media was treatment D which was thought to be due to the interaction between *B.methylotrophicus* with other bacteria. This was allegedly due to the presence of extracellular compounds produced by Bacillus. Other studies also mention that in in vitro, Bacillus probiotics can inhibit the growth of *A. hydrophila* [40]. Extracellular compounds produced by Bacillus include lipase esterase, leucine arylamidase, acid phosphatase, lipase, naphthol-AS-BI-phospholidase, subtilin, coagulin, surfactin, iturin, and bacilysin [41]. It was suspected that surfactin and iturin actively damage the permeability of the membrane of *A.hydrophila*.

Total leukocytes during the study ranged from 66,300–208,700 cells/mm^3. The results of this study indicated that total tilapia leukocytes during the study were in the range of normal values, except on the 13^{th} day which exceeds the normal value. Leukocytes had a number ranging from 20 $x10^3$ cells/mm^3 to 150$x10^3$ cells/mm^3[42]. Leukocyte value of 9-12 cm healthy tilapia was 66,300-112,300 cells/mm^3. Total erythrocytes during maintenance were in the range of 0.3–2.17 x 10^6 cells/mm^3. The results of this study indicated that the number of fish erythocytes in the normal range, except on day 13. Healthy fish erythrocyte values were between 1.57-2.17$x10^6$ cells/mm^3. This value was in the normal range. The total number of normal erythrocytes in teleost fish was 1.05–3.0 × 10^6 cells/mm^3[43].

Hematocrit and hemoglobin values showed values with the same pattern as erythrocyte values when experiencing an increase or decrease. Hematocrit and hemoglobin values in healthy tilapia with size of 9-12 cm were 29.9-32.7% and 5.9-7.4 gdl^{-1}. This value was in the normal range. The average value of hematocrit during the study was 21.8-32.7%. This value was in the normal value, except at the end of the study according to Hrubec *et.al.* [44] that the hematocrit value of normal tilapia was 27-37%. Hematocrit values of teleostei fish are between 20%-30% and hematocrit levels of normal tilapia between 27.3%-37.8% [27].

The hemoglobin value during the study was between 3.1-7.4 g/dl. This value was in the normal range, except on the 13^{th} day. The hemoglobin level of tilapia ranged from 5.05 to 8.33 g/dl [45]. The range of hemoglobin levels in tilapia was 4.50-6.00 gdl^{-1}[46]. The different ranges of hemoglobin levels, due to factors that influence hematological responses in fish include sex, age, size, environment, and physiological conditions [38,47]. Hemoglobin and hematocrit levels were directly proportional to the number of erythrocytes [48].

5. Conclusion

The conclusions that can be drawn from this study were as follows:

1. The addition of *B.methylotrophicus* was able to strongly inhibit *A.hydrophila* and categorized as bactericidal antibiotic with the best density at 10^9 CFU ml^{-1} (24.9 ± 4.2 mm)
2. The use of probiotic candidate bacteria *B.methylotrophicus* in the cultivation medium could increase survival rate until 26,67%;
3. Addition of *B.methylotrophicus* influences the dynamics of tilapia's blood profile, it can inhibit *A.hydrophila* growth rate in media.
4. The clinical symptoms of tilapia that infected by *A. hydrophila* were thinned fins, loose scales, pale fish color, excess mucus, stretched dorsal fin, slow feed response, and swimming unstable.

Acknowledgements. This research is part of the research of candidate probiotic screening on fish production in Central Java by Alfabetian Harjuno Condro Haditomo and team. This research funded by the APBN DPA SUKPA, Faculty of Fisheries and Marine Sciences, Diponegoro University.

References

[1] Hai N V 2015 *Fish & Shellfish Immunology* **45**: 592-597
[2] Feckaninova A, Koscova J, Mudronova D, Popelka P, Toropilova J 2017 *Aquaculture*, **469**: 1–8
[3] Austin and Austin D A 1987. *Bacterial Fish Pathogens Disease in Farmed and Wild Fish*. (Ellis Horwood Limited, England) 364 p
[4] Carnevali O, Maradonna F, Gioacchini G 2017 *Aquaculture* **472**: 144-155

[5] Banerjee G, Ray A K 2017 *Research in Veterinary Science* **115**: 66-77
[6] Haditomo AHC, Sarjito, Desrina, Prayitno SB 2017 10th Symposium On Disease in Asian Aquaculture (DAA 10)
[7] Sharma SCD, Shovon MS, Jahan MGS, Asaduzzaman AKM, Rahman, Biswas KK, Abe N, Roy N 2013 *Journal of Microbiology, Biotechnology and Food Sciences*, **2 (4)**: 2293-2307
[8] Jemil N, Manresa A, Rabanal F, Ayed HB, Hmidet N, Nasri M 2017. *Journal of Chromatography B*, **1060**: 374-386
[9] Yan XH, Song L, Wang G 2011 *Afric J Biotechnol*, **10 (67)**: 15117-15122
[10] Ulkhaq MF, Widanarni, Lusiastuti A M 2014 *Jurnal Akuakultur Indonesia*, **13 (2)**: 105-114
[11] Farias THV, Levy-Pereira N, Alves LDO, Dias DDC, Tachibana L, Pilarski F, Belo M A D A, Ranzani-Paiva MJT 2016 *Animal Feed Science and Technology*, **211**: 137–144
[12] Wahjuningrum D, Hasanah M, Rahman 2016 *Jurnal Akuakultur Indonesia*, **15 (2)**: 108–116
[13] Haditomo AHC, Lusiastuti AM, Widanarni 2016 *Jurnal Saintek Perikanan* **11 (2)**: 111-114
[14] Effendi I 1997 *Biologi Perikanan* (Yayasan Pustaka Nusatama, Yogyakarta) 163 p
[15] Al Harbi AH, Udin NM 2010 *Journal of Applied Aquaculture*, **22**: 187–193
[16] Blaxhall PC, Daisley KW 1973 *Journal of Fish Biology,* **5**: 577–581
[17] Anderson DP and Siwicki AK 1993 2nd Symposium on diseases in Asian Aquaculture "Aquatic Animal Health and the Environment". Phuket, Thailand, p 185-202
[18] Anggriani R, Iskandar, dan Taofiqurohman A 2012 *Jurnal Perikanan dan Kelautan*, **3 (3)**:75-83
[19] Rahayu T 2006 *Jurnal Penelitian Sains dan Teknologi*, **7(2)**: 81-91
[20] El-Kholy A, El-Shinawy S, Meshref A, Korny A 2014 *J. Appl. Environ. Microbiol* **2 (2)**: 53–60
[21] Desriac F, Defer D, Bourgougnon N, Brillet B, Chevalier PL, Fleury Y 2010 *J. Mar. Drugs*, **8**: 1153–1177
[22] Yuliar 2008 *Biodiversitas*, **9 (2)**: 83-86
[23] Huang CH, Ano T, and Shoda M 1993 *J. of Fermentation and Bioengineering*, **76**: 445-450
[24] Lang S and Wagner F 1993 *Bioconversion of Oils and Sugars to Glycolipids* (Biosurfactans Production, Properties, Application. Marcel Dekker. New York) p 346-382.
[25] Hommel R K and Ratledge C 1993 *Biosynthetic Mechanism of Low Molecular Weight Surfactants and Their Precursor Molecules* (Biosurfactans Production Marcel Dekker Inc. New York) 4(3):206-278
[26] Fujaya Y 2004 *Fisiologi Ikan Dasar Pengembangan Teknik Perikanan.* Rineka Cipta, Jakarta, 179 p.
[27] Hardi EH, Pebrianto CA, Hidayanti T, Handayani RT 2011 *Jurnal Kedokteran Hewan* **8 (2)**: 130-134
[28] Hardi E.H, Pebrianto CA, Agustina 2013 *Konferensi Akuakultur Indonesia*. 153-158
[29] Huys G, Kampfer P, Albert MJ, Khun I, Denys R, and Swings J 2002 *Journal of Systematics and Evolutionary Microbiology*, **52**: 705 – 71
[30] Mocanu M, Cristea V, Dediu L, Bocioc E, Grecu RI, Ion S, Vasilean I. 2010 *Lucrari Stiintifice-Sera Zootheniie*, **59**: 258–263
[31] Austin and Austin DA 2007 *Bacterial Fish Pathogens Diseases of Farmed and Wild Fish*. Fourth Edition. (Springer-Praxis Publishing, United Kingdom) 552p
[32] Haditomo A H C, Sarjito, Desrina, Prayitno S B 2018 *IOP Conf. Ser.: Earth Environ. Sci.* **116** 012018
[33] Yousr AH, Napis S, Rusul GRA, Son R 2007 *ASEAN Food Journal*, **14**: 115–122
[34] Chirila F, Fit N, Nadas G, Negrea O, Ranga R 2008 *Isolation and Characterization of an Aeromonas hydrophila Strain in a Carp Cyprynus carpio Toxemia Focus* (Bulletin *UASVM, Veterinary Medicine*) **65**: 244–247.
[35] Kumar MP and Ramulu KS 2013 *International Journal of Food, Agriculture, and Veterinary Sciences,* **3**: 70–75
[36] Pleczar MJ, dan Chan ECS 2009 *Dasar-dasar Mikrobiologi*, Jilid 2 (Universitas Indonesia; Jakarta) p 446-997.

[37] Santoso S 1998 *Jurnal Universitas Sudirman*, **2** (14):5-10.
[38] Haditomo A H C, Sarjito, Desrina, Prayitno S B 2018 *IOP Conf. Ser.: Earth Environ. Sci.* **116** 012018
[39] Hartika R, Mustahal, Putra AN 2014 *Jurnal Perikanan dan Kelautan*, **4 (4)**: 259-267
[40] Sansawat A and Thirabunyanon M 2009 *Maejo International Journal of Science and Technology*, **3**: 77–87
[41] Murilio I, and Villamil L 2011 *Journal of Aquaculture Research and Development*, **07**:1–5
[42] Moyle PB and Cech JJ 2004 *Fishes: An Introduction to Ichtyologi*. 5th ed. USA: Prentice Hall, Inc. USA, 559 p.
[43] Roberts, R.J. 2001. *Fish Pathology*. Bailliere Tindal. London
[44] Hrubec TC, Cardinale JL, Smith SA 2000 *Vet. Clinical Path* **29 (1)**: 7-12
[45] Salasia SIO, Sulanjari D, dan Ratnawati A 2001 *J. Biologi*, **2(12)**:710-723
[46] Dosim, Hardi EH, dan Agustina 2013 *Jurnal Ilmu Perikanan Tropis*, **19(1)**: 1402-2006
[47] Sowunmi AA 2003 *The Zoologist*, **2(1)**: 85-91.
[48] Zuhrawati NA 2014 *Jurnal Medika Veterinaria* **8(1)**: 84-87.