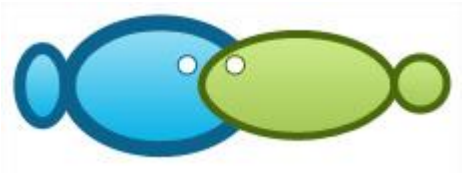

Pathogenicity assay of probiotic-potential bacteria from the Kelabau fish (*Osteochilus melanopleurus*)

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Abstract. This study aimed to evaluate the pathogenicity of probiotic-potential bacteria from the gut of Kelabau fish (*Osteochilus melanopleurus*) collected from Melintang Lake, East Kalimantan Province, Indonesia. Common carp (*Cyprinus carpio*) juveniles were injected with 0.1 mL (10^6 CFU mL^{-1}) bacterial suspension of BP1, BP3, BPs2, BK, *Aeromonas hydrophila*, and phosphate buffer saline, respectively. The injected fish were maintained in 20 L aquarium at a density of 10 individuals/aquarium for 7 days of experiment. Clinical sign, mortality, hematological parameters, histopathology of gills and kidney were observed. The ANOVA test showed that probiotic-potential bacteria from gut of Kelabau fish had a significant effect on the survival rate and hematological parameters of the *C. carpio* juveniles. The infected fish showed changes in clinical signs such as hemorrhage, edema, and ulcer. Histopathological analysis indicated gill hemorrhage, necrosis, hyperplasia, edema and epithelial lifting, kidney necrosis, melanosis and vacuolization in nephron tubular cells. BPs2 isolate provided the best survival rate of 93% and it is a safe option for *C. carpio* as a probiotic-potential bacteria.
Key Words: survival rate, histophatological analysis, common carp, *Aeromonas hydrophila*.

Introduction. Efforts to increase fish production through freshwater fish farming in East Kalimantan Province, especially at Kutai Kartanegara Regency are constant. Kutai Kartanegara Regency was the most important fish producer in East Kalimantan, it delivered 140,133.70 tonnes (BPS 2017), freshwater fish representing 76.28% (106,900.3 tonnes). However, diseases are still a major obstacle in freshwater fish farming in East Kalimantan. The use of local natural ingredients to overcome infectious diseases is an alternative to reduce the use of chemicals and antibiotics. Probiotics are safer ingredients providing benefits like improving the farmed fish health status and being environmentally friendly (Ouwehand et al 2002; Maslowski & Mackay 2010). Screening of potential probiotic bacteria from fish and aquaculture environments in this area has already been carried out and showed positive results (Agustina et al 2010; Agustina et al 2018).

Several types of bacteria obtained from the gut of Kelabau fish (*Osteochilus melanopleurus*) showed the ability to inhibit the growth of two pathogenic bacteria aquaculture fish in the Kutai Kartanegara Regency, namely *Aeromonas hydrophila* and *Pseudomonas* sp. (Agustina et al 2018). Previous tests of several types of bacteria were able to inhibit pathogenic bacteria in vitro, both in the form of whole bacterial cells and cellular product components. Products in the form of probiotic potential bacteria can be produced after a series of follow-up tests in vivo. Pathogenicity assay of intestinal bacteria with demonstrated abilities to inhibit pathogenic bacteria in vitro needs to be further carried out, in order to guarantee the safety of probiotics candidate. Therefore, it is necessary to evaluate their non-pathogenicity in the host (Giri et al 2012).

Based on the background above mentioned, pathogenicity assay was carried out to four selected bacterial isolates from previous tests, namely BP1, BP3, BPs2 and BK with common carp (*Cyprinus carpio*) juveniles as experimental animals, due to its local frequency. This test aimed to evaluate the pathogenicity of the four bacterial isolates and obtain non-pathogenic bacterial isolates that can be used as potentially probiotic bacteria. The health status of *C. carpio* juveniles was checked post infection. The observed parameters were clinical signs, survival rate, hematological parameters and histopathology of the gill and kidney.

Material and Method

Experimental fish. Healthy *C. carpio* juveniles were obtained from a common carp hatchery in the Loa Kulu District, Kutai Kartanegara Regency, East Kalimantan Province, Indonesia. The fish was not showing any signs of disease (inspected through a gross examination of the skin, fins and gills of the respective samples) with no previous history of parasitic and bacterial infection, and having an average weight of 2.6±0.2 g. Fish were maintained in aerated freshwater for 5 days in 40 L glass aquarium. All the fishes were fed three times a day to satiation with pellet containing 38% protein during the acclimatization period.

Preparation of bacteria. Isolates BP1, BP3, BPs2, BK and *A. hydrophila* (AH-1) obtained from a previous test (Agustina et al 2018) were grown for 24 hours in tryptone soy broth media in incubator at 30^{o}C. The harvested cells were washed, resuspended in phosphate buffer saline (PBS). Each bacterial suspension (0.1 mL) contained approximately 10^6 CFU mL^{-1}, as determined by the standard plate count method (Gupta et al 2014).

Determination of possible harmful effects of the bacteria on fish. Each bacterial suspension (0.1 mL) of BP1, BPs2, BP3 and BK were injected intramuscularly into separate groups of 10 *C. carpio*. Another group of 10 fish were injected with phosphate buffer saline as negative control and other 10 fish were injected with 0.1 mL *A. hydrophila* AH-1, as positive control. Once the treatment was performed, each experimental group of fish was reared in 20 L aquarium at a density of 10 fish/aquarium. All experimental fishes were fed three times a day with commercial pellet containing 38% protein for 7 days. Water quality was maintained as follows: temperature 27-29^{o}C, pH 6.4-7.1, dissolved oxygen 6.4-8.1 mg L^{-1} with 7.67-7.95% oxygen saturation and ammonia of 0.0001-0.0035 mg L^{-1}. Half of the water of each aquarium was renewed daily with aerated tap water. Daily observation was recorded for disease incidence and mortality during a 7 days experimental period.

Experimental design. Six experimental treatments were carried out using completely randomized design (CRD). *C. carpio* juveniles were randomly selected and placed into eighteen aquariums with a stocking density of 10 individuals/aquarium. The experiments were run in triplicates. The treatments consisted of an injection of 0.1 mL PBS as a negative control (T0), an injection of *A. hydrophila* AH-1 as a positive control (T1), and four injections of gut bacteria of *O. melanopleurus,* namely BP1 (T2), BPs2 (T3), BP3 (T4) and BK (T5).

Survival rate. Survival rate of the experimental fish (*C. carpio*) was carried out by calculating the number of survivors divided by the total number of initial fish, times 100%. Afterwards, each treatment was tabulated and analysed accordingly.

Haematological parameters. Fish blood parameters were observed three days, five days and seven days post injection. The first step was to take blood samples from the caudal vein using a syringe. Assessments of the blood parameters were done for the total red blood cells and total white blood cells following the Blaxhall & Daisley (1973) procedure, hemoglobin content was assessed using the Sahli method with a haemometer

(Wedemeyer & Yasutake 1977), and hematocrit was assessed according to the method described by Anderson & Siwicki (1995).

Clinical sign. The clinical sign of experimental fish at each treatment were observed post injection treatments. The clinical signs of the experimental fish as a result of treatments were compared with each other. The clinical signs observed included the presence of hemorrhage, edema and ulcer.

Histopathology. A histological examination of the internal organs was performed at the end of the experiment. Samples of gills and kidney were fixed in a buffered neutral formalin solution (10%). The specimens were performed according to Roberts (2001). Tissue sections were stained with hematoxylin-eosin (HE) and observed under Olympus CX23 microscope, photographed using an Olympus SZ-14 camera to study the pathological changes.

Data analysis. The experimental data were tabulated using Microsoft Office Excel 2016 and further analyzed with ANOVA (analysis of variance) using the application of SPSS 14.0 at a 95% confidence level. If the ANOVA showed a significantly different result, a Tukey post hoc test was performed. The parameters of clinical sign, histopathology and water quality were descriptively analyzed.

Results

Survival rate. The survival rate of the experimental fish injected by two gut bacteria, treatments (T3 and T4) were significantly different compared to the positive control (T1) ($p<0.05$) (Table 1). The survival rates of the fish which received T2 and T5 were significantly lower than in the case of the fish treated with T3 and T4. The highest survival rate was obtained by treatment T3 (*C. carpio* juveniles injected by BPs2 isolate, Table 1).

Table 1

Survival rate of *Cyprinus carpio* juveniles one week post pathogenicity test

Treatment	*Cumulative mortality (fish)*							*Survival rate (%)*
	1	*2*	*3*	*4*	*5*	*6*	*7*	
T0	0	0	0	1	1	1	1	96.67 ± 5.77^{b}
T1	7	11	15	17	18	18	18	40.00 ± 10.00^{a}
T2	7	14	17	18	18	18	18	40.00 ± 10.00^{a}
T3	0	1	2	2	2	2	2	93.00 ± 11.55^{b}
T4	1	5	8	9	9	9	9	70.00 ± 10.00^{b}
T5	8	12	15	17	19	19	19	36.67 ± 15.28^{a}

T0 (negative control), T1 (Positive control), T2 (BP1), T3 (BPs2), T4 (BP3), T5 (BK). Data are presented as mean ± SD. The different superscript letter within same column shows a significantly different effect ($p<0.05$).

Total erythrocyte. Total erythrocyte of all treatments tended to decrease post injection except the negative control (To). Total erythrocyte of probiotic-potential bacteria (T2, T3, T4, T5) showed a significant difference ($p<0.05$) compared to the negative control (To) (Table 2). The erythrocyte on treatment T3 (BPs2) was significantly higher ($p<0.05$) compared to the other treatments after three days post injection. However, on day 5 and 7, the negative control (T0) showed the highest total erythrocyte.

Table 2

Total erythrocyte of *Cyprinus carpio* juveniles at the beginning of stocking, day 3, 5 and 7 post bacterial injection

Treatment	Total erythrocyte ($x10^6$ cell mm^{-3})			
	Initial	Day 3	Day 5	Day 7
T0	1.33±0.04	1.58±0.03^{d}	1.72±0.04^{d}	2.03±0.02^{f}
T1	1.33±0.04	1.01±0.04^{c}	0.86±0.01^{b}	0.75±0.01^{b}
T2	1.33±0.04	1.10±0.04^{c}	0.88±0.01^{b}	1.48±0.01^{e}
T3	1.33±0.04	1.66±0.05^{d}	0.84±0.05^{b}	0.87±0.01^{c}
T4	1.33±0.04	0.84±0.02^{b}	1.06±0.02^{c}	1.36±0.01^{d}
T5	1.33±0.04	0.65±0.05^{a}	0.46±0.02^{a}	0.61±0.01^{a}

T0 (negative control), T1 (Positive control), T2 (BP1), T3 (BPs2), T4 (BP3), T5 (BK). Data are presented as mean ± SD. The different superscript letter within same column shows a significantly different effect ($p<0.05$).

Total leukocyte. The total leukocyte in the negative control T0, T3 (BPs2) and T5 (BK) increased significantly ($p<0.05$) compared to T1 (positive control) at day 3 after the bacterial injection. T4 (BP3) showed the highest total leucocyte in day 5 and 7, whilst T3 (BPs2) significantly decreased ($p<0.05$) compared to T1 (positive control) in day 7 post bacterial injection. The total leucocyte of the experimental fish is presented in Table 3.

Table 3

Total leucocyte of *Cyprinus carpio* juveniles at the beginning of stocking, day 3, 5 and 7 post bacterial injection

Treatment	Total leucocyte ($x\ 10^4$ cell mm^{-3})			
	Initial	Day 3	Day 5	Day 7
T0	8.48±0.09	9.24±0.06^{c}	7.84±0.03^{b}	6.46±0.13^{a}
T1	8.48±0.09	6.54±0.11^{a}	8.71±0.12^{c}	12.15±0.05^{d}
T2	8.48±0.09	7.83±0.10^{b}	5.71±0.18^{a}	7.50±0.01^{b}
T3	8.48±0.09	9.59±0.10^{d}	11.35±0.02^{d}	8.47±0.13^{c}
T4	8.48±0.09	6.49±0.04^{a}	11.79±0.18^{e}	15.83±0.26^{e}
T5	8.48±0.09	9.99±0.01^{e}	11.16±0.36^{d}	15.50±0.46^{e}

T0 (negative control), T1 (Positive control), T2 (BP1), T3 (BPs2), T4 (BP3), T5 (BK). Data are presented as mean ± SD. The different superscript letter within same column shows a significantly different effect ($p<0.05$).

Hemoglobin level. The hemoglobin level of the experimental fish in T3 (BPs2) on day 3 post bacterial injection was significantly different ($p<0.05$) compared to the control treatments (T0) (Table 4). In day 5 post bacterial injection, the hemoglobin level of the experimental fish was not significantly different ($p>0.05$) in all the treatments, except at T0 (negative control). The hemoglobin level of T2 (BP1), T3 (BPs2) and T4 (BP3) in day 7 post bacterial injection increased significantly compared to T1 (positive control) (Table 4).

Table 4

Hemoglobin level of *Cyprinus carpio* juveniles at the beginning of stocking, day 3, 5 and 7 post bacterial injection

Treatment	Hemoglobin level (g dL^{-1})			
	Initial	Day 3	Day 5	Day 7
T0	3.97±0.47	3.92±0.14^{c}	4.83±0.14^{b}	7.00±1.00^{d}
T1	3.97±0.47	2.83±0.14^{a}	2.58±0.38^{a}	2.42±0.14^{ab}
T2	3.97±0.47	3.25±0.25^{ab}	3.17±0.29^{a}	3.58±0.29^{bc}
T3	3.97±0.47	3.33±0.14^{b}	2.67±0.29^{a}	3.42±0.14^{bc}
T4	3.97±0.47	2.92±0.14^{ab}	2.92±0.14^{a}	4.04±0.29^{c}
T5	3.97±0.47	2.92±0.14^{ab}	2.75±0.43^{a}	2.17±0.14^{a}

T0 (negative control), T1 (Positive control), T2 (BP1), T3 (BPs2), T4 (BP3), T5 (BK). Data are presented as mean ± SD. The different superscript letter within same column shows a significantly different effect ($p<0.05$).

Hematocrit. Three days after the treatments, T1 (positive control) had a lower level of hematocrit compared to the other treatments. T4 (BP3) and T5 (BK) had a significantly lower level of hematocrit ($p<0.05$) compared to T0 (negative control) (Table 5). The hematocrit of the experimental fish was not significantly different at five days after the treatments ($p>0.05$). Whereas, seven days after the treatments, the hematocrit level of T3 (BPs2) increased compared to the other treatments, except T0 (negative control), yet it was not significantly different ($p>0.05$) (Table 5).

Table 5

The hematocrit level of *Cyprinus carpio* juveniles at the beginning of stocking, day 3, 5 and 7 post bacteria injection

Treatment	*Hematocrit (%)*			
	Initial	*Day 3*	*Day 5*	*Day 7*
T0	22.83±0.50	20.33±1.26^{b}	18.5±1.32^{a}	20.83±0.76^{b}
T1	22.83±0.50	16.33±0.76^{a}	15.17±1.26^{a}	17.33±0.76^{ab}
T2	22.83±0.50	18.33±1.26^{ab}	17.67±1.53^{a}	18.50±0.50^{ab}
T3	22.83±0.50	17.67±1.53^{ab}	17.83±1.44^{a}	19.50±0.87^{ab}
T4	22.83±0.50	16.83±1.26^{a}	16.33±1.26^{a}	17.83±1.04^{a}
T5	22.83±0.50	16.83±0.76^{a}	16.33±1.26^{a}	17.67±1.15^{a}

T0 (negative control), T1 (Positive control), T2 (BP1), T3 (BPs2), T4 (BP3), T5 (BK). Data are presented as mean ± SD. The different superscript letter within same column shows a significantly different effect ($p<0.05$).

Clinical signs. The clinical signs found in the experimental fish injected with *O. melanopleurus* gut bacteria were edema and hemorrhage. In addition to these two signs, fish injected with *A. hydrophila* have also experienced ulcer. In general, the clinical signs were seen 36 hours after treatments. The clinical signs of the experimental fish are presented in Figure 1.

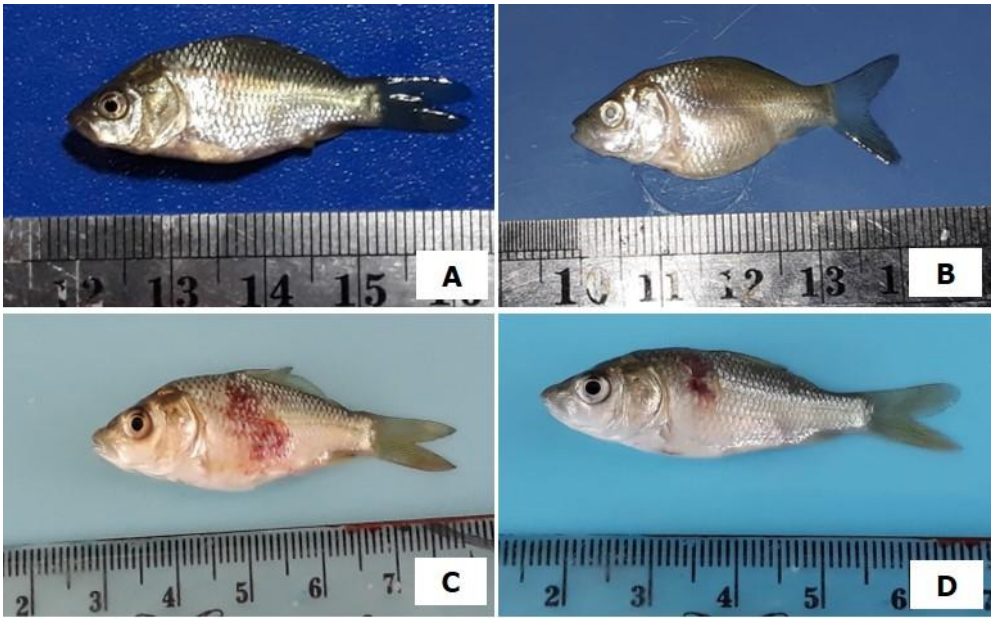

Figure 1. (A) Clinical sign of fish in negative control. (B) Fish injected with gut bacteria of *Osteochilus melanopleurus* (BK) showed edema. (C) Fish injected with gut bacteria of *Osteochilus melanopleurus* (BP1) showed hemorrhage (D) Fish injected with *Aeromonas hydrophila* AH-1 showed ulcer.

Histopathology. The observed histopathological section of gill showed hemorrhage, necrosis, hyperplasia, edema and epithelial lifting, whilst the kidney showed necrosis,

melanosis and vacuolization in the nephron tubular cells. The histopathological section of gill and kidney of the experimental fish are presented in Figure 2 and Figure 3.

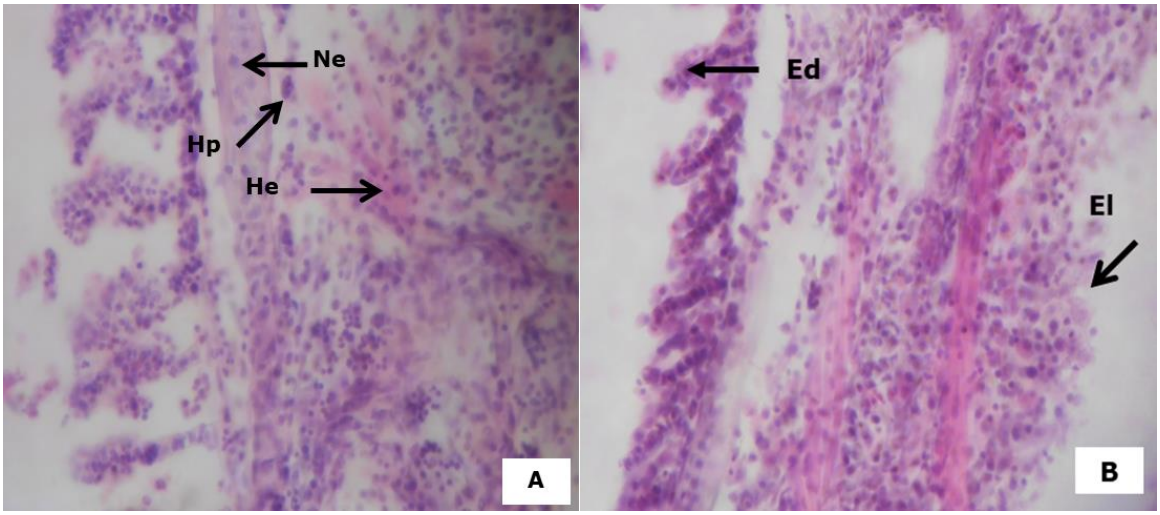

Figure 2. (A) Gill of *Cyprinus carpio* juvenile injected with *Aeromonas hydrophila* AH-1 showed edema (Ed) and epithelial lifting (El) occurred. (B) Gill of *Cyprinus carpio* juvenile injected with gut bacteria of *Osteochilus melanopleurus* (BK) showed hemorrhage (He), necrosis (Ne) and hyperplasia of epithelial cells (Hp) that cover the surface of secondary lamellae (HE stain 100x).

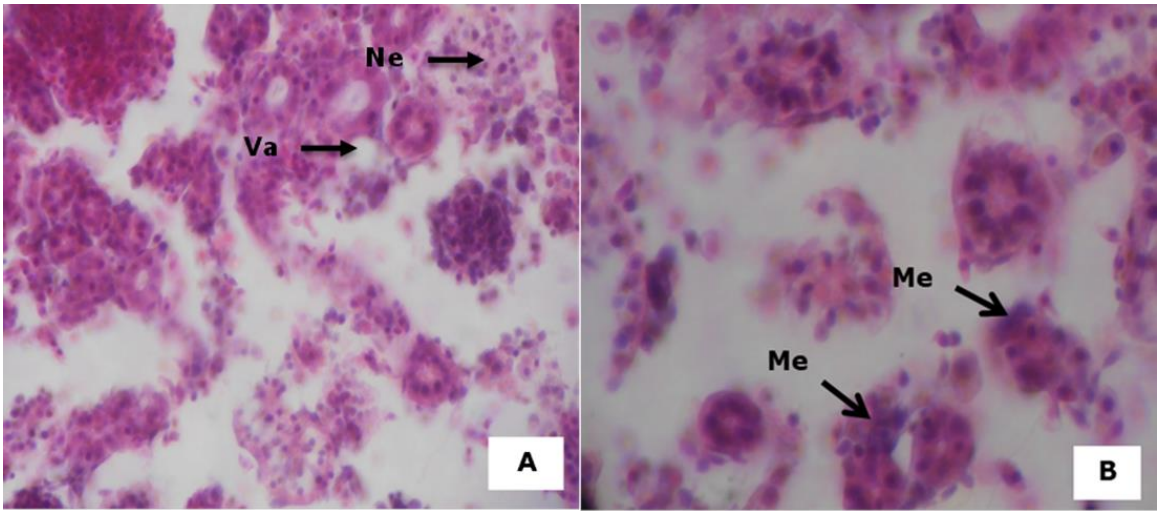

Figure 3. (A) Kidney of *Cyprinus carpio* juvenile injected with *Aeromonas hydrophila* AH-1 showed haemopoietic cells undergo necrosis (Ne) and vacuolization (Va) in nephron tubular cells. (B) Gill of *Cyprinus carpio* juvenile injected with gut bacteria of *Osteochilus melanopleurus* (BK) showed melanosis (Me) in kidney cells (HE stain 100x).

Discussion. The gut bacteria of *O. melanopleurus* that were injected into *C. carpio* juveniles showed different levels of pathogenicity. The T3 (BPs2) gave the best results on the survival rate (93.00±11.55) compared to the positive control (40.00±10.00). The differential levels of resistance in fish could be determined by estimating their survival rate after being infected with bacteria (Wassom & Kelly 1990). In this study, *C. carpio* juveniles had the highest resistance to T3 (BPs2) compared to other treatments especially the positive control (Table 1). A similar result was obtained by Agustina (2007), the probiotic-potential bacteria from catfish (*Clarias* sp.) gut also demonstrated low pathogenicity to catfish juveniles with a survival rate of 85-90%. Moreover, bacteria

of T2 and T5 were suspected to be pathogenic in *C. carpio* juveniles due to a high mortality after injection. Bacteria that have the potential to cause death, both primary and secondary infections, could not be ignored and were thought to cause systemic infections (Burbank et al 2012).

The total erythrocyte of *C. carpio* juveniles decreased after the treatments until the fifth day and increased on the seventh observation day, except on the positive control. The total erythrocyte in the T2, T3, T4 and T5 treatments were significantly higher than in the positive control on day seven after injection (Table 2). The total erythrocyte in the positive control significantly decreased because *A. hydrophila* had hemolysin that could cause erythrocyte lysis (Thune et al 1993; Shao et al 2004). The total leucocytes fluctuated or increased in the infected fish. The total leucocyte on the seventh day of observation decreased in the negative control, T2 and T3 treatments, whereas T1, T4 and T5 were increased (Table 3). Similar to the total leucocytes in T1, T4 and T5 in this study, Li et al (2016) found that the number of white blood cells of grass carp *Ctenopharyngodon idella* increased significantly at 7, 14, 21 days post infection with *A. hydrophila* in the treatment group.

Hemoglobin is a pigment in the erythrocyte and has a function to bind oxygen to be further distributed throughout the body (Reece et al 2014). The hemoglobin level of *C. carpio* juveniles decreased five days after being injected with *O. melanopleurus* gut bacteria T2-T5 (Table 4). The hematocrit levels after the injection with bacteria were not significantly different compared to the positive control (Table 5). In this experiment, a gradual decrease in the total erythrocyte, hemoglobin and hematocrit levels after the injection indicated a damage of the haematopoietic tissues including kidney. This might cause haemorrhagic septicaemia. This could be seen from the haemorrhages on dorsal body parts as well as sero-sanguinous fluid that filled the peritoneal cavity. Decreased blood red cell values, hemoglobin and hematocrit levels indicated that red blood cells were being destroyed by the leucocytosis activity in erythrocytic anemia with subsequent erythroblastosis (Haney et al 1992). Similar to our findings, significant increase in total leucocytes and decrease in the total erythrocyte compared to the control fish was found in *Puntius sarana* infected with *A. hydrophila* (Das et al 2011).

The dorsal part of a *C. carpio* showed hemorrhage 36 hours after treatment. Edema appeared two days after the injection with *A. hydrophila* (positive control) and some *C. carpio* had ulcers on the injection site (Figure 1). Commonly, fish juvenile gills and kidney in this study showed some changes when observed in histological examination. Fish gills showed hemorrhage, necrosis, hyperplasia, edema and epithelial lifting (Figure 2), while kidney experienced necrosis, melanosis and vacuolization in the nephron tubular cells (Figure 3). Changes of clinical signs in this experiment have some similarities, although the degree of severity was observed in the positive control treatments. Clinical signs and histopathological sections were in line with the results of previous studies. Clinical signs such as abdominal ascites (edema), hemorrhage on skin, hemorrhage in gill filaments, swelling and rounded edges also found in the infected tilapia fish with several types of bacteria from the Enterobacteriaceae family (El-Barbary & Hal 2017). Behera et al (2017) further found that the challenged fish with *Acinetobacter baumannii* had loss of mucus and reddish (hemorrhage) lesion near the pectoral fin, however there was no clinical sign in the gills. The histology of experimentally challenged *Labeo rohita* showed hemorrhages and shrunken glomeruli with densely basophilic nuclei in the kidney. Other species have been studied by Rozi et al (2018), they infected *Osphronemus goramy* with five isolates of *A. hydrophila* from virulent and non-virulent strains, and they showed signs of ulcers, hemorrhagic on the base of the fins, body, mouth, and exophthalmia. The histophatology indicated spleen necrosis, picnosis, and inflammatory cells in the kidney.

Factors that play a role in bacterial pathogenicity were the propagation speed of pathogen and host defense against pathogen. Some bacterial extracellular products such as leucosidine and haemolysin were able to induce lysis of the blood cells, and then the bacteria spread throughout the host body to several target organs. Bacteria also have several types of enzymes in their extracellular products such as casein, gelatinase, amylase, lipase, chitinase, collagenase, elastase, hyaluronidase and proteinase that are

able to break down complex compounds into simpler compounds so that the bacteria can easily enter and damage the host cells (Pelcza & Chan 1986; Li et al 2012). The gill and kidney of *C. carpio* juveniles injected with several bacterial isolates of *O. melanopleurus* gut showed some damage and were thought to be caused by some extracellular products from these bacteria.

Bacteria that demonstrated an antagonistic ability to pathogenic bacteria in vitro test were not all safe or non-pathogenic to fish (Burbank et al 2012). In this pathogenicity assay the bacterial isolate BPs2 of *O. melanopleurus* gut bacteria was recommended due to its probiotic potential for further testing in *C. carpio* or other freshwater fish.

Conclusions. From the present study it was concluded that BPs2 bacterial isolate from *O. melanopleurus* gut showed the highest survival rate (93%) after being injected to *C. carpio* juveniles. This bacterium is safe and promising as potential-probiotic bacteria for common carp based on pathogenicity assay. Hematological parameters of *C. carpio* injected with BPs2 were better compared to the positive control and to other treatments. Histopathology of gills and kidney of experimental fish exhibited similar patterns.

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