

Growth performance and immune response of catfish *Clarias* sp. given probiotics *Bacillus megaterium* PTB 1.4 and *Pediococcus pentosaceus* E2211

Kinerja pertumbuhan dan respons imun ikan lele *Clarias* sp. yang diberi probiotik *Bacillus megaterium* PTB 1.4 dan *Pediococcus pentosaceus* E2211

Muhammad Subhan Hamka¹, Anja Meryandini², Widanarni^{1*}

¹Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, Indonesia 16680

²Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor, Indonesia 16680

* Corresponding author: widanarni@yahoo.com

(Received June 2, 2019; Accepted August 27, 2019)

ABSTRACT

Motile aeromonad septicaemia (MAS) in catfish can be done by improving the immune responses through probiotic administration. Co-administering probiotics producing digestive enzymes are expected to have an impact on fish growth. This study was aimed to evaluate the effectivity of probiotics *B. megaterium* PTB 1.4 and *P. pentosaceus* E2211 to improve the growth performance and immune response of catfish infected by *Aeromonas hydrophila*. Catfish with the initial body weight of 7.36 ± 0.21 g were reared in a pond. This study was conducted with five treatments, i. e. K- (without probiotic addition and *A. hydrophila* injection), K+ (no probiotic addition with *A. hydrophila* injection), Bm (*B. megaterium* PTB 1.4 addition and *A. hydrophila* injection), Pp (*P. pentosaceus* E2211 addition and *A. hydrophila* injection), and Bm+Pp (*B. megaterium* PTB 1.4 addition and *P. pentosaceus* E2211 and *A. hydrophila* injection). The study results showed that Bm, Pp, and Bm+Pp treatments were able to improve the growth performance of catfish including digestive enzyme activity, daily growth rate, feed conversion ratio, and final biomass with the best results was Bm+Pp treatment. The immune response of catfish before and after the challenge test showed better survival rate, higher total leukocytes, phagocytic activity, respiratory burst, and lysozyme activity on Bm, Pp, and Bm+Pp treatment ($P < 0.05$) than the control treatment. In conclusion, the combination of probiotics *B. megaterium* PTB 1.4 and *P. pentosaceus* E2211 in feed synergistically improved the growth performance and immune response on catfish against *A. hydrophila* infection.

Keywords: *Aeromonas hydrophila*, catfish, growth performance, immune response, probiotics

ABSTRAK

Motile aeromonad septicaemia (MAS) pada ikan lele dapat dikendalikan melalui respons imun inang dengan pemberian probiotik. Pemberian bersama probiotik penghasil enzim pencernaan diharapkan dapat berdampak pada pertumbuhan ikan. Penelitian ini bertujuan mengevaluasi efektivitas pemberian probiotik *B. megaterium* PTB 1.4 dan *P. pentosaceus* E2211 terhadap kinerja pertumbuhan dan respons imun ikan lele terhadap infeksi *A. hydrophila*. Ikan lele dengan bobot 7.36 ± 0.21 g dipelihara pada kolam beton yang diberi waring. Penelitian ini menggunakan rancangan acak lengkap dengan lima perlakuan, yaitu: K- (tanpa probiotik dan tanpa diinjeksi *A. hydrophila*), K+ (tanpa probiotik dan diinjeksi *A. hydrophila*), Bm (diberi *B. megaterium* PTB 1.4 dan diinjeksi *A. hydrophila*), Pp (diberi *P. pentosaceus* E2211 dan diinjeksi *A. hydrophila*), dan Bm+Pp (diberi *B. megaterium* PTB 1.4 dan *P. pentosaceus* E2211 dan diinjeksi *A. hydrophila*). Hasil penelitian menunjukkan perlakuan Bm, Pp, dan Bm+Pp mampu meningkatkan pertumbuhan ikan lele. Aktivitas enzim pencernaan, laju pertumbuhan harian, rasio konversi pakan, dan biomassa panen dengan hasil terbaik yaitu perlakuan Bm+Pp. Respons imun ikan lele dengan kombinasi probiotik *B. megaterium* PTB 1.4 dan *P. pentosaceus* E2211 mampu meningkatkan sintasan, total leukosit, aktivitas fagositosis, *respiratory burst*, dan aktivitas lisozim, baik pada sebelum dan setelah uji tantang. Pemberian kombinasi probiotik *B. megaterium* PTB 1.4 dan *P. pentosaceus* E2211 pada pakan mampu bekerja sinergis dalam meningkatkan kinerja pertumbuhan dan respons imun ikan lele terhadap infeksi *A. hydrophila*.

Kata kunci: *A. hydrophila*, ikan lele, kinerja pertumbuhan, probiotik, respons imun

INTRODUCTION

Catfish *Clarias* sp. is one of the main aquaculture commodities in Indonesia, especially in Java Island. According to KKP (2017), the catfish production in 2014 reached 485,687 ton and improved until 541,024 ton in 2016. This condition was fulfilled through an intensive culture system, which potentially brought bad impacts, such as water quality reduction and disease attack potential induction. One of the diseases which generally attacks in catfish culture is motile aeromonad septicaemia (MAS) or known as hemorrhagic caused by *Aeromonas hydrophila* infection with 80–100% mortality rate (Asniatih *et al.*, 2013).

A method to overcome this disease generally uses antibiotics, although it can cause some risks, i.e pathogenic bacterial resistance, environmental water pollution, and antibiotic residue on the aquaculture products, therefore their use has been limited (Michael *et al.*, 2014). One alternative solution to resolve these condition is through the probiotic application. Probiotics are defined as microorganisms with the ability of modifying the bacterial composition in the digestive tract of aquatic animals, water, and sediments. Probiotics have been utilized as a feed supplement that can improve the immune response, feed nutrient value, and become biocontrol agent (Flores, 2011).

Some studies reported that the probiotic bacterial roles to induce the host immune response against the pathogenic bacterial infection, such as on striped catfish against *A. hydrophila* (Tamamdusturi *et al.*, 2016) and common carp against *A. hydrophila* (Djauhari *et al.*, 2016). Besides disease, the intensive aquaculture is faced on the minimum commercial feed digestibility rate. The probiotic bacteria can produce some enzymes to improve the feed digestibility, such as amylase, protease, lipase, and cellulase by hydrolyzing the feed nutrients (breaking down carbohydrates, proteins, and lipids into simpler molecules), therefore facilitating the digestion process and nutrient absorption on fish digestive tract (Putra & Widanarni, 2015). Some studies reported that the bacterial probiotic roles are in improving the digestive enzymes and growth, namely on Nile tilapia (Putra & Widanarni, 2015) and vannamei shrimp (Widanarni *et al.*, 2015). The bacterial probiotics used in this study was *Bacillus megaterium* PTB 1.4 isolated from catfish digestive tract (Hamtni *et al.*, 2015), which had been tested effectively to

improve catfish growth performance (Afrilasari *et al.*, 2017). *Pediococcus pentosaceus* E2211 bacteria isolated from corn meal spontaneous fermentation (Rosyidah *et al.*, 2013) and had been tested to improve the catfish resistance against *A. hydrophila* (Turnip *et al.*, 2018). Therefore, the co-application of *B. megaterium* PTB 1.4 and *P. pentosaceus* E2211 bacteria is expected to synergistically improve the growth performance and immune response of catfish against *A. hydrophila* infection. This study was aimed to evaluate the administration effectivity of *B. megaterium* PTB 1.4 and *P. pentosaceus* E2211 probiotics on the growth performance and immune response of catfish against *A. hydrophila* infection.

MATERIALS DAN METHODS

Period and location

This study was conducted in October, 2018 until March, 2019 in the Laboratory of Fish Health, Department of Aquaculture, Faculty of Fisheries and Marine Sciences IPB University, Laboratory of Microbiology, Research Centre for Bioresources and Biotechnology (RCBB) IPB University, and research ponds of Nur Ar Rohman Islamic Boarding School, Tegal Waru Village, Bogor Regency.

Probiotics preparation

Bacterial probiotics used in this study were *B. megaterium* PTB 1.4 isolated by Hamtni *et al.* (2014) from the catfish digestive tract and *P. pentosaceus* E2211 isolated by Rosyidah *et al.* (2013) from corn meal spontaneous fermentation result. These two probiotic bacteria were given a rifampicin antibiotic resistance marker (*B. megaterium* PTB 1.4 Rf^R and *P. pentosaceus* E2211 Rf^R). *B. megaterium* PTB 1.4 Rf^R were cultured on tryptic soy broth (TSB) media and incubated on the waterbath shaker (140 rpm, 29°C) for 12 hours. *P. pentosaceus* E2211 Rf^R were cultured on deMan, Rogosa, and Sharpe broth (MRSB) media and incubated on an anaerobic (37°C) for 18 hours. These two freshly harvested isolates were then centrifuged (9000 rpm) for 5 minutes to obtain probiotic pellets. The probiotic pellets were homogenized in 0.1 mL sterile NaCl 0.85%.

Study design

This study contained five treatments and three replications, namely: (K-) commercial feed given without probiotic addition and *A. hydrophila* injection; (K+) commercial feed given without

probiotic addition, but with *A. hydrophila* injection; (Bm) commercial feed given with 1% *B. megaterium* PTB 1.4 probiotics addition and *A. hydrophila* injection; (Pp) commercial feed given with 1% *P. pentosaceus* E2211 probiotics addition and *A. hydrophila* injection; (Bm+Pp) commercial feed given with 1% *B. megaterium* PTB 1.4 + *P. pentosaceus* E2211 probiotics addition and *A. hydrophila* injection.

Test feed preparation

The feed used was a commercial feed with 39–41% protein content. Feed was added with *B. megaterium* PTB 1.4 Rf^R probiotics, *P. pentosaceus* E2211 Rf^R probiotics, and both probiotic combination, which had been prepared before with each probiotic age of 12 and 18 hours respectively. Feed was then tested the probiotic bacterial viability by calculation using a spread method on tryptic soy agar (TSA) media + 50 µg/mL Rifampicin (for *B. megaterium* PTB 1.4 Rf^R) and deMan, Rogosa and Sharpe Agar (MRSA) + 50 µg/mL Rifampicin (for *P. pentosaceus* E2211 Rf^R). The viability of probiotic bacteria in feed was 10⁶ CFU/g feed.

Pond and rearing media preparation

The pond used in this study was a concrete tank sized 7500×400×60 cm³ given a net sized 100×30×80 cm³ with 15 units. Pond was cleaned first and dried. Water was filled until 30 cm height, then given 227.78 mg/L limestone and filled with water until 50 cm height. Then, added with 55.56 mg/L mollase, 555.56 mg/L manure, 1.1 mg/L yeast, and stood for a week until the water turned into green.

Test animal preparation and rearing

Test fish used were catfish originated from the catfish culturist in Bogor, West Java. Fish with the initial weight of 7.36 ± 0.21 g were acclimatized for one week before used as the test animal and given a standard commercial feed during the acclimatization process. After the acclimatization process had performed, catfish were reared with 30 fish/net density for 30 days and fed three times a day at 04.00, 18.00, and 23.00 (GMT+7) at satiation.

Challenge test

Challenge test was performed on the catfish, a day after reared for 30 days with feed test given. Fish were injected intramuscularly with pathogenic bacterial suspension of *A. hydrophila*

with 0.1 mL 10⁶ CFU/mL per individual using a sterile syringe. The fish treatment on the negative control (K-) were moved into another pond with the same water condition and injected with phosphate buffered saline (PBS). Catfish was then reared again for 10 days given a standard commercial feed and performed an observation on each day.

Parameters

Growth performance

After 30 days of rearing with the test feed, the fish daily growth rate (DGR) and feed conversion ratio (FCR) were calculated based on Akrami *et al.* (2013).

Total probiotic bacteria on the digestive tract

The measurement of total probiotic population was performed after 30 days of rearing using Van Doan *et al.* (2018) method. Catfish digestive tract was taken and measured as much as 0.1 g, then crushed and serially diluted using a sterile PBS solution. The diluted sample was spreaded onto TSA, TSA + 50 µg/mL Rifampicin, and MRSA + 50 µg/mL Rifampicin media to determine the total digestive bacteria, *B. megaterium* PTB 1.4 Rf^R, and *P. pentosaceus* E2211 Rf^R respectively in the digestive tract of catfish on each treatment. The total bacterial colony was calculated and presented in colony forming unit (CFU/g).

Protease and amylase enzyme activity of digestive tract

Protease and amylase enzyme activity in the digestive tract was analyzed after 30 days of rearing. Each fish treatment was taken and measured its digestive tract as much as 1 g, then crushed added with 5 mL phosphate buffer 0.05 M, pH 7.5 and homogenized. Samples were then centrifuged with 6000 rpm at 4°C for 30 minutes. The enzyme crude extracts obtained were tested their activities. Protease enzyme activity was measured based on Walter (1984) method with modification. Amylase enzyme activity was measured based on Bernfeld (1955) method.

Immune response

Total leucocyte measurement followed the procedure of Chen *et al.* (2019). The phagocytic activity was observed through a blood slide of Anderson and Siwicki (1993). The respiratory burst and lysozyme activity were observed following the procedure of Hanif *et al.* (2004).

Total A. hydrophila in the target organ

Total *A. hydrophila* measurement was performed using Van Doan *et al.* (2018) method. Liver and kidney target organ with 0.1 g respectively were crushed and serially diluted using a sterile PBS. The dilution result on each tube was spreaded on Rimler-Shotts (RS) media as much as 0.05 mL to determine total *A. hydrophila* bacteria in the kidney and liver of catfish. The observation was performed on day 30th, 35th, and 40th.

Data analysis

The data obtained were tabulated with WPS Office S spreadsheet 2019. Data were analyzed using a one-way ANOVA (analysis of variance) with SPSS version 16.0 with 95% degree of confidence, when there was a significant difference among treatments, data were tested using a Duncan test.

RESULT AND DISCUSSION**Result***Growth performance*

Survival rate (SR), final biomass (B_t), daily growth rate (DGR), and feed conversion ratio (FCR) of catfish after reared for 30 days are presented on Table 1. The survival rate after 30 days of rearing did not show any differences on all treatments. The initial biomass (B_0) of fish was the same on all treatments, while the final biomass (B_t) of fish on Bm+Pp treatment (1118.54 ± 21.11 g/m²) was higher ($P < 0.05$) than other treatments. The daily growth rate on Bm+Pp treatment (5.46

$\pm 0.17\%$ /day) was also higher ($P < 0.05$) than other treatments. The feed conversion ratio on Bm (0.54 ± 0.01), Bm+Pp (0.60 ± 0.01), and Pp (0.63 ± 0.05) treatment showed lower values ($P < 0.05$) than control. Probiotics addition in feed for 30 days improved DGR, which caused a reduced FCR compared to control.

Total probiotic bacteria in the digestive tract

After 30 days of rearing, total bacteria and probiotic bacteria in the digestive tract are presented on Table 1. Bm (8.41 ± 0.04 log CFU/g), Pp (8.67 ± 0.01 log CFU/g), and Bm+Pp (8.67 ± 0.01 log CFU/g) treatment showed higher total digestive tract bacteria (TB) ($P < 0.05$) than control treatment (7.79 ± 0.00 log CFU/g). Probiotics *B. megaterium* PTB 1.4 Rf^R were only found in treatment Bm and Bm+Pp with the total *B. megaterium* PTB 1.4 Rf^R (TBBm) was 6.60 ± 0.01 log CFU/g and 6.61 ± 0.00 log CFU/g respectively, while on treatment Pp and control were not found probiotics *B. megaterium* PTB 1.4 Rf^R. Moreover, probiotics *P. pentosaceus* E2211 Rf^R (TBPp) were only found on Pp (6.49 ± 0.01 log CFU/g) and Bm+Pp (6.50 ± 0.01 log CFU/g) treatment, while on Bm and control treatment was not found probiotics *P. pentosaceus* E2211 Rf^R.

Protease and amylase enzyme activity in the digestive tract

Probiotics *B. megaterium* PTB 1.4 and *P. pediococcus* E2211 given in feed for 30 days showed higher protease enzyme activity on Bm and Bm+Pp treatment ($P < 0.05$, Figure 1a) than

Table 1. Survival rate (SR), initial biomass (B_0), final biomass (B_t), daily growth rate (DGR), feed intake (FI), feed conversion ratio (FCR), total digestive tract bacteria (TB), total *B. megaterium* PTB1.4 in digestive tract (TBBm) and total *P. pentosaceus* E2211 in digestive tract (TBPp) in catfish after 30 days of rearing.

| Parameter | Treatment | | | | |
|---------------------------|----------------------|---------------------|----------------------|----------------------|-----------------------|
| | K- | K+ | Bm | Pp | Bm+Pp |
| SR (%) | 98.89 ± 1.92^a | 97.78 ± 3.85^a | 94.44 ± 1.92^a | 95.56 ± 3.85^a | 97.78 ± 1.92^a |
| B_0 (g/m ²) | 220.18 ± 8.36^a | 220.89 ± 7.80^a | 222.20 ± 6.25^a | 221.40 ± 1.20^a | 222.51 ± 10.16^a |
| B_t (g/m ²) | 945.38 ± 38.86^a | 915.81 ± 7.21^a | 938.80 ± 16.52^a | 928.27 ± 90.11^a | 1118.54 ± 21.11^b |
| DGR (%/day) | 4.89 ± 0.13^a | 4.82 ± 0.26^a | 4.99 ± 0.11^a | 4.92 ± 0.20^a | 5.46 ± 0.17^b |
| FI (g) | 538.67 ± 0.58^c | 584.70 ± 0.44^c | 395.32 ± 0.21^a | 466.77 ± 0.59^b | 549.07 ± 0.51^d |
| FCR | 0.74 ± 0.03^c | 0.82 ± 0.06^d | 0.54 ± 0.01^a | 0.63 ± 0.05^b | 0.60 ± 0.01^{ab} |
| TB (log CFU/g) | 7.79 ± 0.00^a | 7.84 ± 0.01^b | 8.41 ± 0.04^c | 8.67 ± 0.01^d | 8.67 ± 0.01^d |
| TBBm (log CFU/g) | 0.00 ^a | 0.00 ^a | 6.60 ± 0.01^b | 0.00 ^a | 6.61 ± 0.00^b |
| TBPp (log CFU/g) | 0.00 ^a | 0.00 ^a | 0.00 ^a | 6.49 ± 0.01^b | 6.50 ± 0.01^c |

Note: Different superscript letters on the same line show a significant different treatment influence (Duncan, $P < 0.05$). Values are presented as average and standard deviation, K-: negative control, K+: positive control, Bm: *B. megaterium* PTB 1.4, Pp: *P. pentosaceus* E2211, Bm+Pp: *B. megaterium* PTB 1.4 + *P. pentosaceus* E2211

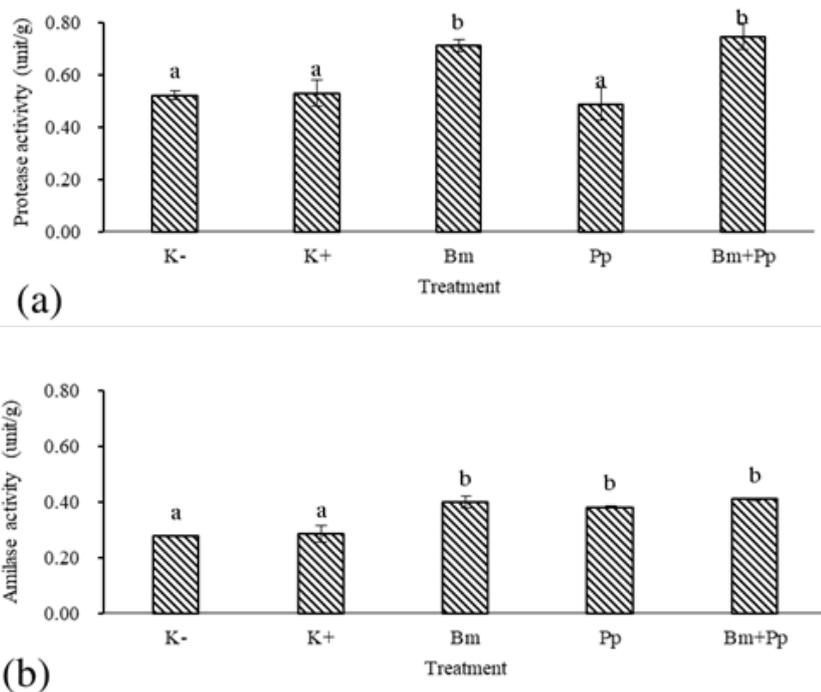


Figure 1 (a-b). Protease and amylase enzyme activity in the digestive tract of catfish after 30 days of rearing. Different letters above the bars show a significant ($P < 0.05$). Negative control (K-), positive control (K+), *B. megaterium* PTB 1.4 addition (Bm), *P. pentosaceus* E2211 addition (Pp), *B. megaterium* PTB 1.4 + *P. pentosaceus* E2211 addition (Bm+Pp).

Table 2. Survival rate (SR) on post-challenge test, total leucocytes (TL), phagocytic activity (PA), respiratory burst activity (RB), and lysozyme activity (LA) of catfish at 0, 30, 35, and 40 days of rearing.

| Parameter | Day | Treatment | | | | |
|--|-----|---------------------------|---------------------------|---------------------------|----------------------------|---------------------------|
| | | K- | K+ | Bm | Pp | Bm+Pp |
| Post-challenge test SR (%) | 40 | 100 ± 0.00 ^c | 43.17 ± 6.67 ^a | 84.69 ± 5.52 ^b | 88.41 ± 1.51 ^b | 87.51 ± 1.87 ^b |
| | 0 | 1.45 ± 0.35 ^a | 1.45 ± 0.35 ^a |
| Total leucocytes (10 ⁴ cells/mm ³) | 30 | 2.08 ± 0.03 ^{ab} | 2.30 ± 0.05 ^{cd} | 2.22 ± 0.02 ^{bc} | 2.44 ± 0.01 ^d | 2.01 ± 0.20 ^a |
| | 35 | 3.21 ± 0.07 ^a | 3.41 ± 0.10 ^a | 3.43 ± 0.31 ^a | 4.49 ± 0.09 ^b | 4.57 ± 0.35 ^b |
| | 40 | 0.99 ± 0.03 ^b | 1.60 ± 0.10 ^c | 0.99 ± 0.04 ^b | 0.78 ± 0.07 ^a | 1.03 ± 0.02 ^b |
| Phagocytic activity (%) | 0 | 10.71 ± 0.71 ^a | 10.71 ± 0.71 ^a |
| | 30 | 11.06 ± 0.94 ^a | 11.96 ± 0.54 ^a | 11.96 ± 0.54 ^a | 12.14 ± 0.62 ^{ab} | 13.39 ± 0.89 ^b |
| | 35 | 14.44 ± 1.92 ^a | 27.58 ± 0.95 ^b | 25.79 ± 0.84 ^b | 33.37 ± 0.89 ^c | 33.97 ± 0.55 ^c |
| | 40 | 14.46 ± 0.31 ^a | 23.89 ± 0.96 ^b | 13.97 ± 0.55 ^a | 14.10 ± 1.32 ^a | 15.56 ± 1.92 ^a |
| Respiratory burst (λ = 630 nm) | 0 | 0.19 ± 0.010 ^a | 0.19 ± 0.010 ^a |
| | 30 | 0.22 ± 0.004 ^a | 0.31 ± 0.002 ^b | 0.29 ± 0.006 ^b | 0.37 ± 0.027 ^c | 0.57 ± 0.011 ^d |
| | 35 | 0.22 ± 0.007 ^a | 0.27 ± 0.004 ^b | 0.33 ± 0.010 ^c | 0.35 ± 0.009 ^c | 0.67 ± 0.017 ^d |
| | 40 | 0.22 ± 0.012 ^a | 0.23 ± 0.009 ^a | 0.27 ± 0.008 ^b | 0.25 ± 0.008 ^b | 0.31 ± 0.018 ^c |
| Lysozyme activity (unit/mL) | 0 | 6.70 ± 0.55 ^a | 6.70 ± 0.55 ^a |
| | 30 | 7.89 ± 0.56 ^a | 11.11 ± 0.00 ^b | 7.67 ± 1.00 ^a | 20.67 ± 0.66 ^c | 26.17 ± 1.72 ^d |
| | 35 | 1.56 ± 0.22 ^a | 2.78 ± 0.48 ^{ab} | 3.33 ± 1.11 ^b | 3.33 ± 1.11 ^b | 5.56 ± 1.11 ^c |
| | 40 | 6.70 ± 0.55 ^c | 4.19 ± 0.17 ^b | 2.22 ± 0.00 ^a | 3.56 ± 0.89 ^b | 4.44 ± 0.02 ^b |

Note: Different superscript letters on the same line show a significant difference (Duncan, $P < 0.05$). Values are presented average and standard deviation. K-: negative control, K+: positive control, Bm: *B. megaterium* PTB 1.4, Pp: *P. pentosaceus* E2211, Bm+Pp: *B. megaterium* PTB 1.4 + *P. pentosaceus* E2211.

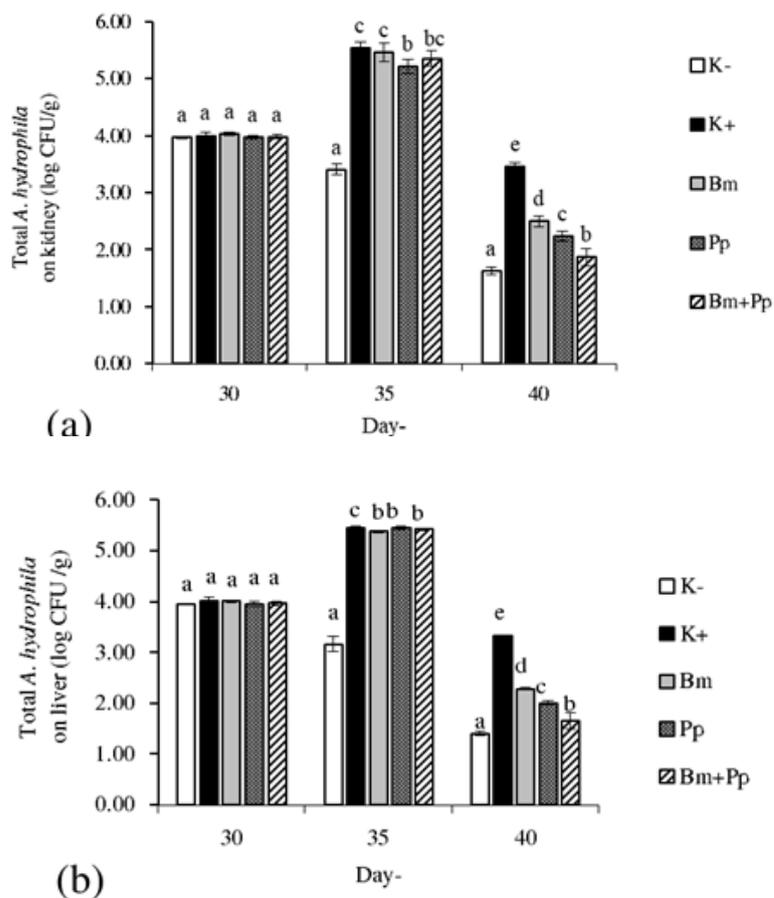


Figure 2 (a-b). Total *A. hydrophila* in the target organs: (a) liver (b) kidney of catfish on the post-challenge test. Different letters above bars show significant difference (Duncan, $P < 0.05$). Negative control (K-), positive control (K+), 1% *B. megaterium* PTB 1.4 addition (Bm), 1% *P. pentosaceus* E2211 addition (Pp), 1% *B. megaterium* PTB 1.4 + 1% *P. pentosaceus* E2211 addition (Bm+Pp).

other treatments (Pp and control). Meanwhile, amylase enzyme activity on Bm, Pp, and Bm+Pp treatment was higher ($P < 0.05$, Figure 1b) than control treatment, however were insignificantly different among those probiotic treatments.

Immune response

Probiotics *B. megaterium* PTB 1.4 and *P. pediococcus* E2211 given in feed influenced the survival rate (SR) on the post-challenge test and blood profiles of catfish as presented on Table 2. Total leucocytes (TL), phagocytic activity (PA), respiratory burst activity (RB), and lysozyme activity (LA) had different values on each treatment, which represented a fish health status alteration. The SR value of Bm ($84.69 \pm 5.52\%$), Pp ($88.41 \pm 1.51\%$), and Bm+Pp ($87.51 \pm 1.87\%$) treatment was higher ($P < 0.05$) than the positive control ($43.17 \pm 6.67\%$) on the 40th day (10 days of post-challenge test).

Total leucocyte of catfish on 0 day in all treatments showed the same value, i.e $1.45 \pm$

0.35×10^4 cells/mm³. After 30 days of rearing, total leucocytes was increased and Pp treatment ($4.49 \pm 0.09 \times 10^4$ cells/mm³) was higher ($P < 0.05$) than other treatments. Increased total leucocytes at the 35th day was higher than at the 30th day on all treatments, and the highest value was found on Bm+Pp ($4.57 \pm 0.35 \times 10^4$ cells/mm³) followed with Pp treatment ($4.49 \pm 0.09 \times 10^4$ cells/mm³) ($P < 0.05$) compared to other treatments. Decreased total leucocytes happened at the 40th day in all treatments with the lowest value ($P < 0.05$) was found on Pp treatment ($0.78 \pm 0.07 \times 10^4$ cells/mm³).

The phagocytic activity of catfish at 0 day showed the same value on each treatment, i.e $10.71 \pm 0.71\%$. Phagocytic activity of catfish was increased at 30th day on all treatments, and Bm+Pp treatment ($13.39 \pm 0.89\%$) showed higher FA value than other treatments. Phagocytic activity was continuously increased at the 35th day on all treatment, Bm+Pp ($33.97 \pm 0.55\%$) and Pp ($33.37 \pm 0.89\%$) treatment was higher ($P < 0.05$) than

other treatments. Decreased phagocytic activity happened on all treatments at the 40th day, except on the positive control. The highest phagocytic activity value ($P < 0.05$) was shown on the positive control with $23.89 \pm 0.96\%$ compared to the other treatments.

Respiratory burst activity of catfish at 0 day showed no difference ($P > 0.05$) with 0.19 ± 0.01 . Increased respiratory burst happened at the 30th day, Bm+Pp treatment (0.57 ± 0.011) showed higher value ($P < 0.05$) than other treatments. Respiratory burst was continuously increased at the 35th day as Bm+Pp treatment (0.67 ± 0.017) showed higher value ($P < 0.05$) than other treatments. Respiratory burst activity was decreased at the 40th day as Bm+Pp treatment (0.31 ± 0.018) had lower value than other treatments.

The lysozyme activity of catfish at 0 day showed no different value on each treatment with 6.70 ± 0.55 unit/mL. Lysozyme activity was increased on all treatments after 30 days of rearing, the highest value ($P < 0.05$) was presented on Bm+Pp treatment with 26.17 ± 1.72 unit/mL. Lysozyme activity was decreased at 35th day on all treatments as Bm+Pp treatment showed the highest value ($P < 0.05$) compared to other treatments with 5.56 ± 1.11 . Increased lysozyme activity occurred at the 40th day on Pp (3.56 ± 0.89 unit/mL) and positive control treatment, while Bm+Pp (4.44 ± 0.02 unit/mL) and Bm (4.19 ± 0.17 unit/mL) treatment showed decreased values.

Total A. hydrophila calculation result on the target organ

Total *A. hydrophila* on the target organs of catfish on the post-challenge test is presented on Figure 2. Total *A. hydrophila* pathogenic bacteria in kidney and liver of catfish were the same on all treatments with approximate value of 3.97 ± 0.02 until 4.03 ± 0.03 log CFU/g at the 30th day. Total *A. hydrophila* pathogenic bacteria in liver and kidney of catfish at the 35th day was increased on all treatments, except the negative control (approximately 3.94 ± 0.01 until 4.00 ± 0.08 log CFU/g). Total *A. hydrophila* pathogenic bacteria at the 40th day was gradually decreased, Bm+Pp treatment showed the lowest value in kidney (1.87 ± 0.15 log CFU/g) and liver (1.65 ± 0.16 log CFU/g), and significantly different ($P < 0.05$) against other treatments.

Discussion

The results showed higher daily growth rate,

feed conversion ratio, final biomass, digestive enzyme activities (protease and amylase) on Bm, Pp, and Bm+Pp treatment than control. The existence of *B. megaterium* PTB 1.4 in the digestive tract of catfish (Table 1) was suspected to influence this condition. This followed the study results of Afrilasari *et al.* (2017) who used *B. megaterium*, Putra and Widanarni (2015) and Hauville *et al.* (2016) who used *Bacillus* sp., as well as Ferguson *et al.* (2010), Neissi *et al.* (2013), Adel *et al.* (2017), Valipour *et al.* (2018), and Gong *et al.* (2019) who reported the use of *P. acidilactici* and *P. pentosaceus* as digestive enzyme producing probiotics to improve the growth performance of catfish, green terror, grass carp, kutum, Nile tilapia, common carp, and vannamei shrimp. Fish feed contains sufficiently high proteins as the main components in feed and energy source for fish. Feed that enters the fish digestive tract will be degraded by the digestive enzymes. *B. megaterium* PTB 1.4 has a proteolytic characteristic, which can secrete a protease enzyme to hydrolyze the peptide bonds in proteins to become oligopeptides and amino acids. Moreover, *B. megaterium* PTB 1.4 also secretes amylase enzyme to breakdown carbohydrates into maltose and glucose. The best results were shown on Bm+Pp treatment with the highest daily growth rate ($5.46 \pm 0.17\%/hari$) and final biomass (1118.54 ± 21.11 g/m²) of catfish and significantly different ($P < 0.05$) compared to other treatments. This was suspected due to the induced exogenous and endogenous enzyme activity in the digestive tract, therefore improving the daily growth rate and final biomass of catfish. The substrate availability in digestive tract can improve the exogenous and endogenous enzyme activities in digestive tract. Feed that enters into the fish digestive tract will be digested with the help of exogenous enzymes from the probiotic bacteria and endogenous enzymes produced by the fish (Afrilasari *et al.*, 2017). The existence of endogenous and exogenous enzyme collaboration causes the feed is easily digested and absorbed by the fish body, therefore improving the growth performance of catfish after given probiotic treatments (Afrilasari *et al.*, 2017).

The survival rate of catfish on the post-challenge test period indicated higher value ($P < 0.05$) on Bm, Pp, and Bm+Pp treatment than control. Normal physiological response and immune response against the disease attack can be detected by measuring the fish blood profiles. Based on the blood profile measurements (Table

2), the total leucocytes (TL), phagocytic activity (PA), and respiratory burst activity (RB) improved after 30 days of rearing, and continuously improved at the 35th day of post-challenge test. This condition indicated that catfish fought against the pathogenic bacterial attack. The total leucocytes on Bm, Pp, and Bm+Pp treatment were reduced at the 40th day, which indicated that catfish was gradually recovered.

The immune system on fish body contains two types, namely non-specific and specific immune. A non-specific immune system is divided into the first defense system or physical defense (such as scales and mucus) and second defense or humoral mechanism with plasmatic character, such as lysozyme, interferon, etc. (Uribe *et al.*, 2011). The addition of *B. megaterium* PTB 1.4 and *P. pentosaceus* E2211 through feed could improve the value of TL, PA, and RB. The Bm+Pp treatment showed the highest TL ($4.57 \pm 0.35 \times 10^4$ sel/mm³), PA ($33.97 \pm 0.55\%$), and RB (0.67 ± 0.017) at the 35th day of post-challenge test, then reduced at the 40th day. Leucocytes play important roles on the non-specific immune system during the inflammation occurred and their number can become a fish health status indicator. TL and PA alteration happened when the fish suffered from an infection or stress condition. The increased TL during post-infection is related to the inflammatory response mediated by leucocytes to face the pathogenic bacterial infection. High TL after given probiotics for 30 days and 35 days after challenge test showed that the leucocytes were produced in high quantity to fight against the infection of *A. hydrophila*.

Leucocytes are blood cells that involves in the phagocytosis process. phagocytosis is the basic body defense and plays a role in limiting and breaking the foreign cells. The role of phagocytosis is mediated by monocytes, neutrophils, and macrophages as presented from the value of phagocytic activity (Awasthi *et al.*, 2013). Phagocyte cells will kill the bacteria by producing reactive oxygens during the respiratory burst process (Uribe *et al.*, 2011). Sugiani *et al.* (2013) stated that the higher respiratory burst activity, the greater production of free radicals used to fight the pathogens. The improved leucocytes, phagocytic activity, and respiratory burst indicated the health status of fish after given probiotics, namely Bm, Pp, and Bm+Pp treatment at the 35th day compared to 30th day and 0 day was higher ($P < 0.05$) than control. Total leucocytes, phagocytic activity, and respiratory

burst activity at the 40th day on all probiotic treatments were decreased compared to the positive control. This condition showed that fish had undergone a recovery period towards the normal condition. This followed the study results of Bunnoy *et al.* (2019), who used *Acinetobacter* KU011TH on catfish and Silarudee *et al.* (2019) who used *Lactobacillus plantarum* CR1T5 on striped catfish could induce the respiratory burst activity compared to control.

Lysozyme is an important defense molecule in the form of proteins that involves in the non-specific immune, including in fish and has a lytic activity against Gram positive and negative bacteria, besides activating the complement and phagocytosis system. Moreover, lysozyme can also hydrolyze the N-acetylmuramate and N-acetylglucosamine acid, which are the peptidoglycan layer components in the bacterial cell wall (Chen *et al.*, 1996; Awasthi *et al.*, 2013). Based on the measurement result of catfish lysozyme, all treatments showed increased value at the 30th day with the highest value was on Bm+Pp treatment (26.17 ± 1.72 unit/mL). This happened as *B. megaterium* and *P. pentosaceus* bacteria as Gram positive bacteria stimulated the lysozyme activity by producing bactericidal enzymes to fight against the disease agents. Nayak (2010) reported that probiotics either in single or combination could trigger the lysozyme level on Teleost fish. The lysozyme value was then gradually low at th 35th day with 5.56 ± 1.11 unit/mL and 4.44 ± 0.02 unit/mL at the 40th day. This condition was suspected due to the bacterial pathogen existence in the pond which had been attacked the catfish until the 30th day, as seen from high number of *A. hydrophila* in the target organs (either in kidney or liver), therefore most enzymes produced on the lysozyme activity were utilized in lyzing the pathogenic bacteria, then finally the lysozyme activity was gradually reduced at th 35th and 40th day. Nasrullah *et al.* (2019) reported that the lysozyme activity in kidney and liver organ of catfish after infected with *A. hydrophila* was increased and reached an optimum point at the 12th hour, then gradually decreased until the normal condition.

The probiotic administration is known to be capable of improving the host resistance, as seen from continuously induced blood profile value and reduced total *A. hydrophila* pathogenic bacteria in the target organ. Catfish had undergone a recovery period at the 40th day with the best condition was obtained from Bm+Pp treatment. This was

shown on the low total *A. hydrophila* pathogenic bacteria ($P < 0.05$) in kidney (1.87 ± 0.15 log CFU/g) and (1.65 ± 0.16 log CFU/g) compared to other treatments. This condition was as same as the study results of Chen *et al.* (2018), who utilized probiotics *Paenibacillus ehimensis* on Nile tilapia against the infection of *A. hydrophila* and *Streptococcus iniae*. One of the probiotic actions to inhibit the pathogen infection was by improving the host immune response through the stimulation of body non-specific and cellular immunity (Fyzul *et al.*, 2014). This condition happened as probiotics and components or their products are interacted with the gut associated lymphoid tissue (GALT) to induce the host immune response (Dimitrioglou *et al.*, 2011).

Based on the immune response parameter results above containing total leucocytes, phagocytic activity, respiratory burst activity, lysozyme activity, and total *A. hydrophila* pathogenic bacteria in the target organs of catfish before and after the challenge test, the probiotic treatments were generally better than the control treatment, especially on Bm+Pp treatment, which was the combination of probiotics *B. megaterium* PTB 1.4 and *P. pentosaceus* E2211.

CONCLUSION

A combined probiotics of *B. megaterium* PTB 1.4 and *P. pentosaceus* E2211 given in feed can work synergistically to improve the growth performance and immune response of catfish against *A. hydrophila* infection.

REFERENCES

- Adel M, Yeganeh S, Dawood MAO, Safari R, Radhakrishnan S. 2017. Effect of *Pediococcus pentosaceus* supplementation on growth performance, intestinal microflora and disease resistance of white shrimp, *Litopenaeus vannamei*. *Aquaculture Nutrition* 23: 1401–1409.
- Afrilasari W, Widanarni, Meryandini A. 2017. Effect of probiotic *Bacillus megaterium* PTB 1.4 on the population of intestinal microflora, digestive enzyme activity and the growth of catfish *Clarias* sp.. *Hayati Journal of Biosciences* 23: 1–5.
- Akrami R, Iri Y, Rostami HK, Mansour MR. 2013. Effect of dietary supplementation of fructooligosaccharide (FOS) on growth performance, survival, *Lactobacillus* bacterial population and hemato-immunological parameters of stellate sturgeon *Acipenser stellatus* juvenile. *Fish & Shellfish Immunology* 35: 1235–1239.
- Anderson DP, Siwicki AK. 1993. Basic hematology and serology for fish health programs. Di dalam: Shariff M, Arthur JR, Subasinghe JP, editor. *Diseases in Asian Aquaculture II*. 1993 Oktober 25-29: Phuket, Thailand. Manila (PH): Fish Health Section, Asian fisheries society. 185–202.
- Asniatih M, Idris K, Saibu. 2013. Pathological change of African catfish *Clarias gariepinus* infected by *Aeromonas hydrophila*. *Jurnal Mina Laut Indonesia* 3: 13–21.
- Awasthi A, Rathore G, Sood N, Khan MY, Lakra WS. 2013. Establishment of a leukocyte cell line derived from peritoneal macrophages of fish, *Labeo rohita* (Hamilton, 1822). *Cytotechnology* 67: 85–96.
- Bernfeld P. 1955. Amylases α and β . *Method Enzymol* 1: 149–158.
- Bunnoy A, Na-Nakorn U, Srisapoom P. 2019. Probiotic effects of a novel strain, *Acinetobacter* KU011TH, on the growth performance, immune responses, and resistance against *Aeromonas hydrophila* of bighead catfish (*Clarias macrocephalus* Günther, 1864). *Microorganism* 7: 613–643.
- Chen SC, Yoshida T, Adams A, Thompson KD, Richards RH. 1996. Immune response of rainbow trout to extracellular products of *Mycobacterium* sp. *Journal of Aquatic Animal Health* 8: 216–222.
- Chen SW, Liu CH, Hu SY. 2018. Dietary administration of probiotic *Paenibacillus ehimensis* NPUST1 with bacteriocin-like activity improves growth performance and immunity against *Aeromonas hydrophila* and *Streptococcus iniae* in Nile tilapia *Oreochromis niloticus*. *Fish & Shellfish Immunology* 84: 695–703.
- Chen H, Yuan G, Su J, Liu X. 2019. Hematological analysis of *Ctenopharyngodon idella*, *Megalobrama amblycephala* and *Pelteobagrus fulvidraco*: Morphology, ultrastructure, cytochemistry and quantification of peripheral blood cells. *Fish & Shellfish Immunology* 90: 376–384.

- Dimitroglou A, Merrifield DL, Carnevali O, Picchiatti S, Avella M, Daniels C, Gúroy D, Davies SJ. 2011. Microbial manipulations to improve fish health and production - a Mediterranean perspective. *Fish & Shellfish Immunology* 30: 1–16.
- Djauhari R, Widanarni, Sukenda, Suprayudi MA, Zairin-Jr M. 2016. Characterization of *Bacillus* sp. NP5 and its application as probiotic for common carp *Cyprinus carpio*. *Research Journal of Microbiology* 11: 101–111.
- Ferguson RMW, Merrifield DL, Harper GM, Rawling MD, Mustafa S, Picchiatti S, Balcazar JL, Davies SJ. 2010. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia *Oreochromis niloticus*. *Journal of Applied Microbiology* 109: 851–862.
- Flores ML. 2011. The use of probiotic in aquaculture: an overview. *International Research Journal of Microbiology* 2: 471–478.
- Fyzul AN, Harbi AHA, Austin B. 2014. Review: developments in the use of probiotics for disease control in aquaculture. *Aquaculture* 431: 1–11.
- Gong L, He H, Dongjie L, Cao L, Khan TA, Yanping L, Lifei P, Liang Y, Xuezhi D, Yunjun S, Youming Z, Ganfeng Y, Shengbiao H, Liqiu X. 2019. A new isolate of *Pediococcus pentosaceus* (SL001) with antibacterial activity against fish pathogens and potency in facilitating the immunity and growth performance of grass carps. *Frontiers in Microbiology* 10: 1–14
- Hamtini, Widanarni, Meryandini A. 2015. Isolation and selection of *Bacillus* sp. from catfish *Clarias* sp. as a potential probiotic. *Jurnal Biologi Indonesia* 11: 11–19.
- Hanif A, Bakopoulos V, Dimitriadis GJ. 2004. Maternal transfer of humoral specific and non-specific immune parameters to sea bream *Sparus aurata* larvae. *Fish & Shellfish Immunology* 17: 411–435.
- Hauville MR, Zambonino-Infante JL, Bell JG, Migaud H, Main KL. 2016. Effects of a mix of *Bacillus* sp. as a potential probiotic for florida pompano, common snook and red drum larvae performances and digestive enzyme activities. *Aquaculture Nutrition* 22: 51–60
- KKP [Pusat Data Statistik dan Informasi Kementerian Kelautan dan Perikanan]. 2017. Produksi dan Nilai Produksi Budidaya Lele Menurut Provinsi 2003–2016.
- Michael ET, Amos SO, Hussaini LT. 2014. A review on probiotics application in aquaculture. *Fisheries and Aquaculture Journal* 5: 111–113.
- Nasrullah H, Nababan YI, Yanti DH, Hardiantho D, Nuryati S, Junior MZ, Ekasari J, Alimuddin. 2019. Identifikasi dan analisis ekspresi gen lisozim tipe-C dan tipe-G pada ikan lele dumbo setelah infeksi *Aeromonas hydrophila*. *Jurnal Akuakultur Indonesia* 18: 1–10.
- Nayak SK. 2010. Review probiotics and immunity: a fish perspective. *Fish & Shellfish Immunology* 29: 2–14.
- Neissi A, Rafiee G, Nematollahi M, Safari O. 2013. The effect of *Pediococcus acidilactici* bacteria used as probiotic supplement on the growth and non-specific immune responses of green terror *Aequidens rivulatus*. *Fish & Shellfish Immunology* 35: 1976–1980
- Putra AN, Widanarni. 2015. Screening of amyolytic bacteria as candidate of probiotic in tilapia *Oreochromis* sp.. *Research Journal of Microbiology* 10: 1–13.
- Rosyidah E, Meryandini A, Sunarti TC. 2013. The use of lactic acid bacteria and cellulolytic bacteria to improve the chemical properties of corn flour. *Makara Journal of Science* 17: 75–80.
- Silarudee S, Tongpim S, Charoensri N, Doolgindachbaporn S. 2019. Effect of a probiotic *Lactobacillus plantarum* CR1T5 dietary supplements on non-specific immunity in black eared catfish (*Pangasius larnaudii*). *Journal of Pure and Applied Microbiology* 13: 289–296
- Sugiani D, Sukenda, Harris E, Lusiastuti AM. 2013. Vaksinasi ikan tilapia *Oreochromis niloticus* menggunakan vaksin monovalen dan bivalen untuk pencegahan penyakit *motile aeromonas septicemia* dan streptococcosis. *Jurnal Riset Akuakultur* 8: 229–239.
- Tamam dusturi R, Widanarni, Yuhana M. 2016. Administration of microencapsulated probiotic *Bacillus* sp. NP5 and prebiotic Mannan Oligosaccharide for prevention of *Aeromonas hydrophila* infection on *Pangasianodon hypophthalmus*. *Journal of Fisheries and Aquatic Science* 11: 67–76.
- Turnip ER, Widanarni, Meryandini A. 2018. Seleksi bakteri asam laktat sebagai probiotik dan evaluasi kinerjanya pada ikan lele gnotobiotik *Clarias* sp. *Jurnal Akuakultur Indonesia* 17: 68–80.

- Uribe C, Folch H, Enriquez R, Moran G. 2011. Innate and adaptive immunity in teleost fish: a review. *Veterinarni Medicina* 56: 486–503.
- Valipour AR, Shahraki NH, Biria HA. 2018. Effect of probiotic *Pediococcus acidilactici* on growth and survival of kutum *Rutilus kutum* fingerlings. *Iranian Journal of Fisheries Sciences* 17: 35–46
- Van Doan H, Hoseinifar SH, Khanongnuch C, Kanpiengjai A, Unban K, Van Kim V, Srichaiyo S. 2018. Host-associated probiotics boosted mucosal and serum immunity, disease resistance and growth performance of Nile tilapia *Oreochromis niloticus*. *Aquaculture* 491: 94–100.
- Walter HE. 1984. Method with hemoglobin, casein and azocoll as substrate. Di dalam: Bergmeyer HU, editor. *Methods of Enzymatic Analysis*. Ed ke-3. Weinhem (JE): Verlag Cheime. hlm 270-277
- Widanarni, Nopitawati T, Jusadi D. 2015. Screening of probiotic bacteria candidates from gastrointestinal tract of Pacific white shrimp *Litopenaeus vannamei* and their effects on the growth performances. *Research Journal of Microbiology* 10: 145–157.