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Selection of lactic acid bacteria as a probiotic and evaluated its performance on gnotobiotic catfish *Clarias* sp.

Seleksi bakteri asam laktat sebagai probiotik dan evaluasi kinerjanya pada ikan lele gnotobiotik *Clarias* sp.

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ABSTRACT

This study aimed to select lactic acid bacteria (LAB) as a potential probiotic that producing anti-microbial compounds in order to treat motile aeromonads septicemia disease caused by *Aeromonas hydrophila* on catfish *Clarias* sp. and evaluated its performance on gnotobiotic catfish. The in vitro assay was done to select several LAB isolates based on antagonistic activity against pathogenic bacteria. The selected isolate was tested in vivo to observe their ability to improve growth performances of catfish. The study was conducted with five treatments consists of K- (normal catfish without addition probiotic, without challenge test), K+ (normal catfish without addition of probiotic, with challenge test), Np (normal catfish with addition of probiotic and challenge test), G (gnoto catfish without addition of probiotic, with challenge test), and Gp (gnoto catfish with addition of probiotic and challenge test). The results showed that the addition of *Pediococcus pentosaceus* E2211 as selected probiotic could increase survival rate, specific growth rate, and immune response towards infection of *A. hydrophila*. The best survival rate after challenge test was obtained in Np and Gp treatments (88.46%), followed by G treatment (65.38%), while the K+ was only 53.84%. The conclusion of this study was *P. pentosaceus* E2211 potentially used as a probiotic candidate for normal and gnotobiotic catfish. The presence of normal microflora with *P. pentosaceus* E2211 in Np treatment showed the best probiotic performance with daily growth rate 3.28%, feed conversion ratio 1.79, and total intestinal bacteria reached 108 CFU/mL significantly different from other treatments (P<0.05).

Keywords: *Aeromonas hydrophila*, catfish, LAB, probiotic, screening

ABSTRAK

Tujuan penelitian ini adalah menyeleksi bakteri asam laktat (BAL) sebagai probiotik potensial penghasil senyawa antimikrob guna menanggulangi penyakit motile aeromonad septicemia akibat *Aeromonas hydrophila* pada ikan lele *Clarias* sp. dan evaluasi kinerjanya pada ikan lele gnotobiotik. Pengujian in vitro dilakukan untuk menyeleksi beberapa isolat BAL sebagai kandidat probiotik berdasarkan aktivitas antagonis terhadap bakteri patogen. Isolat terpilih kemudian diuji in vivo untuk mengetahui kemampuannya dalam meningkatkan performa tumbuh ikan lele. Penelitian ini menggunakan rancangan acak lengkap dengan lima perlakuan, yaitu: K- (lele normal tanpa probiotik dan tanpa tanpa diuji tantang), K+ (lele normal tanpa probiotik dan diuji tantang), Np (lele normal diberi probiotik dan diuji tantang), G (lele gnoto tanpa probiotik dan diuji tantang), dan Gp (lele gnoto diberi probiotik dan diuji tantang). Hasil penelitian menunjukkan pemberian probiotik terpilih BAL *Pediococcus pentosaceus* E2211 mampu meningkatkan sintasan, laju pertumbuhan, dan respons imun ikan lele terhadap infeksi *A. hydrophila.* Sintasan terbaik pascauji tantang diperoleh pada perlakuan Np dan Gp yaitu sebesar 88,46%, diikuti perlakuan G sebesar 65,38%, sementara pada K+ hanya mencapai 53,84%. Kesimpulan dari penelitian ini ialah isolat BAL terpilih *P. pentosaceus* E2211 berpotensi sebagai kandidat probiotik untuk ikan lele normal dan lele gnotobiotik *Clarias* sp. Keberadaan mikroflora normal yang berasosiasi dengan P. pentosaceus E2211 pada perlakuan Np menunjukkan kinerja probiotik terbaik dengan nilai laju pertubuhan harian 3,28%, rasio konversi pakan 1,79 dan total bakteri usus mencapai 108 CFU/mL yang berbeda signifikan dibanding perlakuan lainnya (P<0,05).

Kata kunci: *A. hydrophila*, BAL, ikan lele, probiotik, seleksi

INTRODUCTION

Catfish *Clarias* sp. is one of the leading commodities in freshwater fish with high demand and is already reared by the society. That high demand will be demanding in increasing catfish production to fulfill the demand (KKP, 2014). The increasing production of catfish intensively often experiences a variety of problems, one of them is disease attack. The disease that often attacks the catfish is motile aeromonads septicemia (MAS) disease, also known as redness blotches disease caused by *A. hydrophila* bacterial infection (Kusdarwati *et al*., 2017). Lukistyowati and Kurniasih (2012) stated that *A. hydrophila* affected freshwater fish rearing and often cause outbreaks of disease with a high mortality rate (80–100%) in a short time (1 to 2 week). According to Anyanwu *et al.* (2015), the mortality range of catfish infected by *A. hidrophila* could reach 90% of mortality. Wahjuningrum *et al.* (2013) also reported that the infection *of A. hydrophila* on the density of 10^4 CFU/mL can cause a mortality of juvenile catfish up to 50% of the population.

The use of antibiotics for MAS disease control continuously appear a variety of negative impacts such as the pathogen resistance and the accumulation of toxic residues (Hao *et al*., 2014). Therefore, it takes an alternative prevention for MAS disease that is effective, secure, inexpensive, environmental friendly and has no negative effects on consumers.

An effort that can be done to prevent the MAS disease is using lactic acid bacteria (LAB) as probiotics candidate (control agent) on the catfish (Suskovic *et al*., 2010). The species of lactic acid bacteria that has been widely utilized and has been successfully commercialized as a probiotic were from the genera of Lactobacillus and Bifidobacterium. This two species of bacteria has known as probiotics candidate that are safe and able to produce bacteriocin metabolite to inhibit the growth of pathogens and increase the host immunity (Hegarty *et al*., 2016; Gomez *et al*., 2016) therefore it can be utilized as an alternative to replace the antibiotics.

Lactic acid bacteria are the gram-positive bacteria, non-spore forming bacteria, anaerobic or facultative, rod-shaped or rounded bacteria, and produce lactic acid as an end product during the fermentation of carbohydrates process (Quinto *et al*., 2014). Tambekar and Bhutada (2010) added that the agent of anti-bacteria like lactic acid can inhibit the growth of pathogenic bacteria because it can lower the pH, therefore, the pathogenic bacteria is difficult to survive. In this study, the performance of LAB as probiotics was observed in normal catfish and gnotobiotic catfish (Situmorang *et al*., 2014). According to Martin *et al*. (2016), the gnotobiotic animal is a group of germ-free animal and has no microorganisms living both inside and outside their body to control the microbial specifically so that the interactions between the host and the microbial can be seen clearly. In gnotobiotic catfish, the existence of good bacteria both outside their body and in their digest tracts was being abolished or reduced by using various types of antibiotics, therefore, the role of probiotic bacteria can be evaluated. This study aimed to select a few isolates of the LAB and to evaluate its performance through the catfish feed in normal catfish and in gnotobiotic catfish as an effort to control the *A. hydrophila* infection.

MATERIALS AND METHODS

Isolate of bacteria

The bacteria that used in this study were LAB isolates of *Lactobacillus plantarum* E1211, *Pediococcus pentosaceus* E1222, *P. pentosaceus* E2211, *P. pentosaceus* E5, *P. pentosaceus* E7, and *P. pentosaceus* E8, were a collection from Laboratory of Animal Biotechnology, Research Center for Biological Resources and Biotechnology (PPSHB), Bogor Agricultural University and *A. hydropliha* isolates that obtained from a collection of Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University.

The pathogenicity test

The pathogenicity test carried out through *in vitro* test in blood agar plate. LAB isolates streaked on blood agar plate and incubated at 27–28 °C for 24 hours. The pathogenicity marked from the formation of a clear zone around the colonies. Isolates that did not form a clear zone around the colonies were then selected to be tested further.

Antagonistic test of lactic acid bacteria towards *Aeromonas hydrophila*

The inhibited activity of LAB isolates towards *A. hydrophila* known on its size of the inhibited zone that was formed by using agar spot

assay (Klose *et al.*, 2010). Each of LAB isolate inoculated in 50 mL of liquid medium of man ragosa sharpe (MRS) and incubated at 37 °C for 10 to 14 hours (the optical density reached 0.8), then it centrifuged at 7000 rpm for 20 minutes to obtain free cell supernatant, it neutralized the pH (pH reached 6.5) by using 1 N NaOH. Meanwhile, the culture of *A. hydrophila* (with a density of 10^6 CFU/mL) poured in trypticase soy agar (TSA) plate. After it became solid, agar spot assay on the media is made by using sterilized straw (with autoclave for 15 minutes at 121 °C and pressure of 15 lbs). Then free cell supernatant of volume 25, 50, 75, and 100 µL inserted into each spot and incubated at 30 °C for 24 hours, observed, and measured the inhibited zones by using the ruler.

The growth curve

The bacterial growth curve aimed to find out the bacterial growth phases and to determine the harvesting time of the cells. A single colony bacteria inoculated into 20 mL of sterile media MRSB and incubated at 37 °C for 10 minutes. Then 10 mL of the cultured bacteria inoculated into 90 mL of sterile MRSB medium and incubated at 37 °C. The growth of bacteria was observed every one hour by measuring the optical density based on absorbance value using a spectrophotometer at a wavelength of 620 nm.

The mixture of selected lactic acid bacteria in feed and the viability test

The feed sterilized using an autoclave at 121 °C and 15 lbs of pressure for 15 minutes. The density of probiotics that added to feed was 10^8 and 10^{10} CFU/mL. The determination of probiotics density was based on the results of the calculation of total plate count (TPC) after several hours of incubation, then was diluted using phosphate buffer saline (PBS). The selected lactic acid bacteria inoculated on MRSB medium and incubated at 37 °C. The cultured bacteria was then centrifuged at 7000 rpm for 20 minutes, the cells were taken and re-suspended with PBS in which obtained the concentrations of 10^8 and 10^{10} CFU/mL. As many as 1% of probiotics and 2% of egg whites as the adhesive sprayed onto the feed. After LAB was being mixed in feed, the viability of the bacteria tested through it took 1 g of feed to be diluted into 9 mL of 0.85% NaCl and 0.1 mL of bacterial suspension streaked on MRS solid medium and incubated for 24 hours at 37 °C, and then the colonies of the bacteria were calculated.

The determination of lethal dose 50 (LD_{50}) of *Aeromonas hydrophila* towards the catfish

The pathogenicity of *A. hydrophila* in catfish was tested by determining the lethal dose 50 (LD_{50}) that indicated by the bacterial density caused 50% of fish mortality. The catfish injected with *A. hydrophila* (10^5, 10^6, 10^7, and 10^8 CFU/mL) through intramuscular injection and reared for 10 days in the aquarium (sizing 60×30×40 cm, containing 10 L of water with a stocking density of each aquarium was 10 fishes). The observation is done every day to obtain the mortality of fish. The concentration of *A. hydrophila* that was obtained from the LD_{50} test as much as 10^6 CFU/mL used as the infection dose of *A. hydrophila* upon the challenge test of catfish.

The experimental preparation of normal catfish and gnotobiotic catfish

The experimental fish that used in this study had two treatments i.e. normal catfish and gnotobiotic catfish. The preparation for gnotobiotic catfish was done by adding different types of antibiotics into the water, they were 250 mg/L of ampicillin, 125 mg/L of rifampicin, and 250 mg/L of chloramphenicol for the reduction of bacteria in the outside part of the fish body. After that, the fish was being fast for 24 hours, until then they were given feed that containing 100 µg/mL of rifampicin for the reduction of the bacteria in the digestive tract for 4–5 days (Afrilasari, 2017). The utilization of rifampicin is based on the sensitivity result of catfish's intestinal bacteria that showed the intestinal bacteria of catfish tend to be sensitive to rifampicin more than other antibiotics.

The preparation of experimental feed

The feed used for the study were commercial pellet 781 (from the production of PT. Central Pangan Pertiwi) with 31–33% of protein content. The probiotic dose that used was as much as 1% in 100 g of total feed (Wang, 2007). The feed preparation included spraying the probiotics as much as 1% with a density of 10^8 CFU/mL in the feed and the addition of 2% of egg white as an adhesive.

In vivo test in catfish

Catfish that used in the study was from catfish farmers in Dramaga, Bogor. Fish acclimated to experimental condition for a week before being given the treatment. The average weight was 8.76 g and reared in aquariums sizing 60×30×40 cm^3 with a density of 15 fishes/aquarium. The

fish reared for 30 days and fed three times a day (in the 08.30 a.m, 14.00 a.m and 19.30 pm, night) with feeding rate of 4–5% of fish biomass. The water quality in aquarium kept through siphoning and changing the water as much as 30% from total volume of the aquarium every two days.

After 30 days of rearing, the challenge test was done through intramuscular injection of *A. hydrophila* into the fish body. The fish rearing during the challenge test lasted for 10 days. This study consisted of five treatments, they were K- (normal catfish fed with feed without probiotic and not tested challenge), K+ (normal catfish fed with feed without probiotic and tested challenge), Np (normal catfish fed with feed added with probiotics and tested challenge), G (gnotobiotic catfish fed with feed without probiotic and tested challenge), and Gp (gnotobiotic catfish fed with feed added probiotics and tested challenge).

Growth parameters

Survival rate (SR)

The survival rate of the experimental fish was observed at the end of the study that is calculated by using the following formula:

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

SR = Survival rate (%)
Nt = The total fish at the end of the study
No = The total fish at the initiation of the study

Specific growth rate (SGR)

The specific growth rate of the fish calculated based on the average weight of every 10 days during the fish rearing. The specific growth rate of the fish is calculated by using the following formula:

$$SGR\ (\%) = \left[\sqrt[t]{\frac{Wt}{Wo}} - 1\right] \times 100$$

SGR = Specific growth rate (%/day)
Wt = The average weight of fish at the end of study (g)
Wo = The average weight of fish at the initiation of the study (g)
t = Days of rearing

Feed conversion ratio (FCR)

The feed conversion ratio was observed during the rearing by calculating the amount of eaten feed. The feed conversion ratio is calculated using the following formula (Huisman, 1987):

$$FCR = \frac{F}{Wt + D - Wo}$$

FCR = Feed conversion ratio
F = The amount of eaten feed (g)
Wt = The average weight of fish at the end of study (g)
Wo = The average weight of fish at the initiation of the study (g)
D = The average weight of dead fish (g)

The total amount of intestinal bacteria

The total amount of bacteria in the digestive tract of fish is calculated by using TPC (total plate count) method. The calculation of the total amount of intestinal bacteria was done after 30 days of rearing and after 10 days of challenge test. The digestive tract of catfish as much as 1 g crushed and dissolved in 10 mL of sterile PBS, then it homogenized by using vortex and 0.85% NaCl for serial dilution. The sample that has been diluted was scattered on TSA plate for obtaining total intestinal bacteria, in the other hand, MRSA used for total intestinal of LAB and MRSA with 50 µg/mL addition of rifampicin used for total probiotics bacteria, is incubated for 24 hours at room temperature, and the total of colonies was counted.

Hematological parameters

Total erythrocyte

Blood samples were sucked by using a pipette with a scale of 0.5 and was added with Hayem's solution up to 101. The blood and Hayem's solution in the pipette were shacked for 3–5 min in eight form until it got the homogeneity. The first drop was disposed and the following was dropped slowly to haemocytometer, covered it with a cover glass, and observed it by using a microscope. The calculation was performed in five different points of view of haemacytometer. The total erythrocyte is calculated using the following formula:

$$RBC = \frac{\text{Counted red blood cell}}{5} \times \frac{1}{\text{The square volume}} \times \frac{1}{\text{Dilution factor}}$$

Total leukocyte

Blood samples were sucked by using a pipette with a scale of 0.5 and was added with Turk's solution up to the scale of 11. The blood and Turk's solution in the pipette were shacked for 3–5 min in eight form until it got the homogeneity. The first drop was disposed and the following was dropped slowly to haemocytometer, covered it with a cover

glass, and observed it by using a microscope. The calculation was performed in five different points of view of haemacytometer. The total leukocyte is calculated using the following formula:

$$\text{WBC} = \frac{\text{Counted white blood cell}}{5} \times 25 \times \frac{1}{\text{The square volume}} \times \text{Dilution factor}$$

Haemoglobin level

Haemoglobin level (Hb) measurement was carried out through Sahli method that converts blood into haematic acid (Gillet *et al*., 2009). Blood samples were sucked by using Sahli pipette to scale of 0.2 mL, transferred it into Hb-meter tube that already filled with HCl 0.1 N to scale of 10 (red colored), stirred it, and rested for 3–5 min. Afterward, an aquades was added until the blood and HCl had the similar color to the indicator of Hb-meter. The scale of matched color signified the haemoglobin level.

Phagocytic activity of fish

The phagocytic activity could be obtained from the capability of leukocyte cell to phagocyte the pathogen cell. Firstly, *Staphylococcus aureus* bacteria were cultured in tryptic soy broth (TSB) medium for 18 hours. After that, 10^6 CFU/mL of bacteria and fish blood was taken as much as 50 µL of it, mixed it, and was incubated at room temperature for 20 min. Then the blood dropped slowly into slide glass, withdrawn it by using another slide glass with forming 45°, and finally drained it well. That sliding glass soaked with methanol for 10 min and drained it again before being wash with Giemsa solution for 10 min. After all, it rinsed gently by using aquades and drained it again. Furthermore, it was observed by using a microscope. The phagocytic activity is calculated using the following formula:

$$\text{Phagocytic index} = \frac{\sum \text{Phagocytizing cell}}{\sum \text{Phagocytic cell}} \times 100$$

Data analysis

Survival rate data, specific growth rate, feed conversion ratio, the total number of bacteria, and the hematological parameters analyzed by analysis of variance (ANOVA). If it was a significant difference, it continuously performed with Duncan tested on α = 0.05. The data were analyzed by using SPSS 15.0 software.

RESULTS AND DISCUSSIONS

Pathogenicity test

In vitro tests in blood agar medium aimed to find out the haemolysis capability of bacteria isolates (Mangunwardoyo *et al*., 2010). The result of pathogenicity test from the sixth LAB isolates showed that all of that isolates in this study did not form a clear zone which on blood agar medium (Figure 1). It showed that all of six bacteria isolates were nonhaemolytic bacteria (able break down the erythrocytes). Suardana *et al*. (2014) reported that in some isolates, the formation of clear zone indicates the virulence of bacteria to break down the erythrocytes by extracellular bacteria protein substance known as haemolysin as an expression of its phenotype. The formation of widely clear zones on the blood agar medium showed the β-haemolysis activity in bacteria isolates. The result of this study was in accordance with Damodharan *et al.* (2015) that stated LAB does not generally have a haemolytic activity.

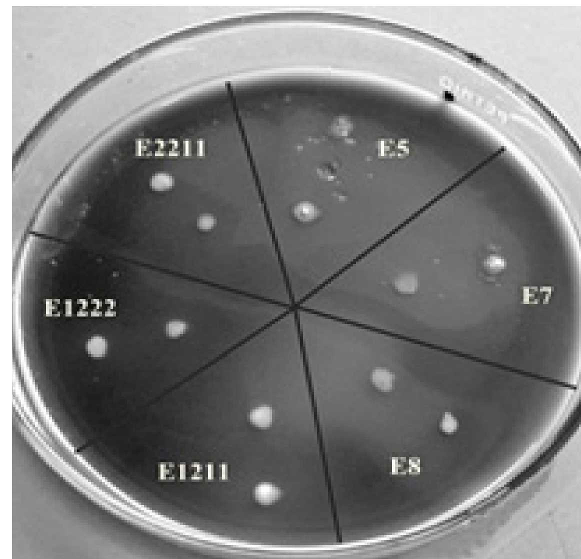

Figure 1. The result of pathogenicity test from six LAB isolates on blood agar medium

Antagonistic test of lactic acid bacteria towards *Aeromonas hydrophila*

The antagonistic test aims to find out at the ability of LAB isolates in producing antimicrobial compounds to inhibit the growth of *A. hydrophila*. The LAB isolates can produce various protein (polypeptide) substance that has antimicrobial activity i.e bacteriocin during the growth period (Zacharof & Lovitt, 2012). The antagonistic activity was characterized by the formation of inhibitory zones around the spot on media after it giving LAB free cell supernatant at low pH and neutral pH (pH 6.5). The LAB free cell supernatant treatment that was not neutralized aimed to maintain acidic conditions of organic acid compounds, while the neutralized

Table 1. The index of supernatant inhibitor zone from the six isolates of lactic acid bacteria against *Aeromonas hydrophila*

No.	The isolates of lactic acid bacteria	Supernatant initial pH	The index of the inhibitory zone	
			Initial pH	Neutralized pH (pH 6.5)
1.	*Lactobacillus plantarum* E1211	3.96	0.184	-
2.	*Pediococcus petosaceus* E1222	3.92	0.164	-
3.	*Pediococcus pentosaceus* E2211	3.91	0.269	-
4.	*Pediococcus pentosaceus* E5	3.90	0.307	-
5.	*Pediococcus pentosaceus* E7	3.99	0.195	-
6.	*Pediococcus pentosaceus* E8	4.03	0.130	-

treatment of free cell supernatant aimed to eliminate the antibacterial influence of organic acid so that the active antibacterial substance was only the organic compounds (Puspita, 2011). All of LAB isolates showed the inhibitory activity against *A. hydrophila* towards low pH, yet it has not shown the inhibitory activity at neutralized pH (Table 1). This was likely due to bacteriocin that produced was still not able to inhibit *A. hydrophila* in the density of 10^6 CFU/mL or the experimental bacterial isolates tend to produce organic acids. According to Khunajakr *et al.* (2008), LAB strain has the ability to produce organic acids that can potentially apply as probiotics. Lactic acid is able to weaken the permeability of bacteria especially in outer membranes of gram-negative bacteria through breaking down its part.

The repeat test conducted on selected three LAB isolates rely on its inhibitor zone. It was done by increasing the viscosity of supernatant concentration that produced on neutralized pH conditions. The observation result from the index of supernatant inhibitor zone against *A. hydrophila* showed the highest was LAB *P. pentosaceus* E2211, moreover, it was selected as probiotics candidate for catfish on the next experimental stages (Table 2). Rosyidah *et al.* (2013) reported that *P. pentosaceus* E2211 is one of LAB that was successfully isolated from the fermentation process (immersion) of corn spontaneously for 48 hours. *P. pentosaceus* E2211 is a gram-positive bacteria with coccus-shaped and has negative catalase.

The growth curve of selected bacterial isolates

The curve of growing bacteria was observed through the increasing of optical density towards the time (Figure 2). The growth curves of *P. pentosaceus* E2211 has a lag phase (adaptation) about two hours of incubation period. The adaptation phase is an adjustment phase of the bacteria that already moved into a new medium with any different substrate and conditions of its environment (White, 2007). The time of this phase was varied, depending on the ability of bacteria to adapt to the surrounding environment. The second hour until the sixth hour of the cells entered the log phase (exponential) was characterized by increasing the OD significantly, the seventh hour to 18th-hour, the cells entered stationary phase and at 19th-hour and so on, the cells experienced the death phases, which was characterized by decreasing value of OD. Exponential phase is the phase of bacteria active growth, moreover, the cells mass increase exponentially towards the time, while the stationary phase is a stop phase of cells growth (White, 2007) with bacterial growth that tends to go slow and remain. The death phase is the phase of decreasing the number of bacterial cells because the bacteria dies after running out

Table 2. The index of supernatant inhibitor zone towards increasing the viscosity 25 times from the three isolates of lactic acid bacteria against *Aeromonas hydrophila*

The supernatant concentration (pH 6.5) (µL)	The index of inhibitor zone		
	Pediococcus pentosaceus E2211	*Pediococcus pentosaceus* E5	*Pediococcus pentosaceus* E7
25	-	-	-
50	0.285	-	0.093
75	0.427	0.233	0.312
100	0.458	0.312	0.457

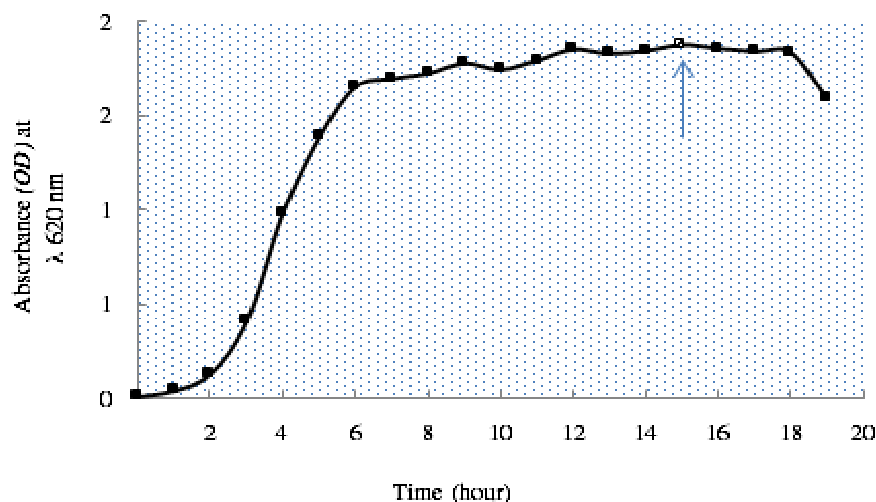

Figure 2. The growth curve of selected isolate *P. pentosaceus* in MRSB medium for 19 hours at 37 °C. The arrow showed the limit of bacterial growth zenith

of some nutrients and it is on the incompatible environmental conditions i.e an existence of toxic matter from bacterial metabolism during incubation (Klose *et al.,* 2010). According to the growth curve that obtained from this study, the cells harvesting time for the next cells production on the next stages are performed on the exponential phase, or at the sixth hour.

The viability of probiotic bacteria in fish feed

The ability of probiotic bacteria to survive is known through testing the viability of probiotic in fish feed. The viability of fresh probiotics cultures during preparation and storage is the main thing in probiotic preparation (Wang *et al.,* 2008) moreover the probiotics can be applied and provide benefits to its host. The density of probiotics *P. pentosaceus* E2211 that added into the feed for viability test is 10^8 and 10^{10} CFU/ml (Table 3). Afrilasari *et al*. (2017) reported that the results of viability test of probiotic *Bacillus megaterium* PTB 1.4 (the density of 10^8 CFU/mL) that added into feed is able to survive with a cell density of 10^6 CFU/mL and significantly affect the total intestinal bacteria of catfish, while Zare *et al. (*2015) reported that the addition of probiotics *Pediococcus acidilactici* with density of 10^{10} CFU/ml also give the significant effects towards the total number of LAB in intestine.

The viability of LAB isolates *P. pentosaceus* E2211 was still well even though it was either experienced a decreasing in the total number of bacteria after mixing feed. The decrease in the total number of bacteria due to the number of probiotics that added to feed as much as 1% in 100 g gained 0.01% of probiotics. It showed that *P. pentosaceus* can be applied as a probiotic in fish through the fish feed. *P. pentosaceus* has been widely applied as a feed additive in the United States, around Europe, China, Thailand, Australia, and New Zealand (Lim & Tan, 2009). According to the results of the test, the viability of *P. pentosaceus* E2211 in feed, the density of *P. pentosaceus* E2211 that used in the in vivo test of catfish was 10^8 CFU/mL.

The determination of lethal dose 50 (LD_{50}) of *Aeromonas hydrophila* towards the catfish

The virulence of *A. hydrophila* can be known through the LD_{50} value is produced, it was the observation to a dose that capable to kill 50% of the fish population. According to Rey *et al.* (2009), the infection of *A. hydrophila* can cause clinical symptoms after several hours of infection and the mortality began after 7 hours. The infection further will cause mortality after 12–24 hours. As for the densities of *A. hydrophila* used in the observation of LD_{50} test towards the catfish were 10^5, 10^6, 10^7, and 10^8 CFU/mL (Table 4).

Table 3. The viability of *Pediococcus pentosaceus* E2211 in fish feed

Treatments	The initial addition of bacteria (CFU/mL)	The total probiotic bacteria after 30 min of mixing (CFU/mL)
The 1st viability test	10^8	5.4×10^6
The 2nd viability test	10^{10}	1.3×10^9

Table 4. The determination of lethal dose 50 (LD_{50}) of *Aeromonas hydrophila* towards the catfish

Densities (CFU m/L)	Dead	Live	Mortality ratio	Accumulation			
				Dead	Live	Mortality ratio	% of mortality
10^8	10	0	10/10	21	0	21/21	100.00
10^7	8	2	8/10	11	2	11/13	84.61
10^6	3	7	3/10	3	9	3/12	25.00
10^5	0	10	0/10	0	19	0/19	0.00

According to the fish mortality pattern, after the fish injected with *A. hydropila*, it can be seen that the higher the density of *A. hydrophila* is injected then so does the mortality rate of fish. The density of bacteria that cause 50% of mortality in fish population within 5 days of incubation was 10^6 CFU/mL. According to LD_{50} test that has been performed, it obtained that the concentration of *A. hydrophila* was 10^6 CFU/mL used for challenge test. Triyaningsih *et al.* (2014) stated that the LD_{50} value can indicate the level of virulence from the bacteria. Bacteria that has a LD_{50} value between $10^{4.5}$–$10^{5.5}$ CFU/mL belongs to a high virulent group of bacteria; an LD_{50} value between $10^{5.5}$–10^7 CFU/mL belongs to a virulent group of bacteria; and LD_{50} more than 10^7 CFU/mL is an avirulent group of bacteria.

The growth performance of catfish

The growth performance of catfish after in vivo test was showed through its survival rate after 30 days of rearing, the specific growth rate, and the feed conversion ratio. The survival rate of catfish at all the treatments during the 30 days of rearing was 100%. This showed that the treatment of gnotobiotic and probiotic during this study was safe for catfish. Probiotic treatment produced the highest SGR compared to other treatments, but the FCR was not significantly different (P>0.05) with other treatments (Figure 3; Table 4). The specific growth rate of all the treatments tended to experience an increasing at 10, 20, and 30 days of rearing. During 10 days of rearing, the SGR value of gnotobiotic catfish (G and Gp) was lower than the controls (K- and K+). This indicated that the gnotobiotic catfish was still in its recovery time after various antibiotics treatment so that the result of the SGR value was still low. At 20th day of rearing, there was a significant difference between the Np treatment (2.70 ± 0.24) with K- treatment (2.12 ± 0.14). The highest SGR value after 30 days of rearing was Np treatment that significantly different (P < 0.05) with four other treatments.

Np treatment was a treatment feed with the addition of probiotic *P. pentosaceus* E2211. This is indicated that the presence of probiotics *P. pentosaceus* E2211 was capable to associate with intestinal microflora in increasing the nutrients absorption thus increasing the specific growth rate of fish. The improvement of fish growth after probiotic treatment occurred due to the increase of appetite and digestibility of fish. The increasing of fish digestibility that fed with probiotics occurred because the probiotic synthesized the extracellular enzymes such as protease, amylase, and lipase, and a gr owth factor such as vitamins, fatty acids, and amino acids (Sahu *et al.,* 2008). Xing *et al.* (2013) reported that fed supplementation of *P. pentosaceus* (10^9 CFU/g) in cobia fish (*Rachycentron canadum*) for two weeks showed a better growth than the control.

The higher growth in probiotic treatment associated with intestinal bacteria of experimental fish. The highest total number of intestinal bacteria, total LAB, and total probiotic of *P. pentosaceus* E2211 obtained at Np treatment that significantly different (P<0.05) with the other treatments (Table 6). This was because in addition to the capability of bacteria to colonize optimally, the probiotic *P. pentosaceus* E2211 was also capable to associate with LAB microflora in the catfish's intestine so it experienced an increasing the amount of LAB in the intestine and suppressed the pathogenic bacteria. According to Hegarty (1999) the ability of probiotics in improving the performance of the growth of its host can be seen from the microbial community in its intestine. The balance of the intestinal microflora can cause the probiotic reported that the addition of the LAB probiotic *Lactobacillu*s sp. is able to increase the number of intestinal bacteria at the end of rearing.

The existence of LAB probiotic can balance the digestion microbes through growth induction of good bacteria and inhibit pathogenic bacteria through the of lactic acid production that is bactericidal (Askarian *et al.,* 2011). The LAB group can also produce acetic acid, hydrogen

Table 5. The survival rate and feed conversion ratio of catfish feed after 30 days of rearing

Treatments	Survival rate (%)	Feed conversion ratio
K-	100 ± 0.00[a]	1.95 ± 0.09[a]
K+	100 ± 0.00[a]	1.99 ± 0.06[a]
Np	100 ± 0.00[a]	1.78 ± 0.13[a]
G	100 ± 0.00[a]	2.03 ± 0.24[a]
Gp	100 ± 0.00[a]	1.94 ± 0.09[a]

Note: The numbers that followed with the same superscripts in the same column showed significantly different according to Duncan test at a confidence level of α 0.05

peroxide, and bacteriocin that can suppress the growth of competitors bacteria (Castex *et al.,* 2008; Maeda *et al.,* 2013). Moslehi *et al.* (2016) reported that *P. pentosaceus* is capable to colonize well in the digestive tract of Siberian sturgeon (*Acipenser baerii* Brandt, 1869) with the highest total of *P. pentosaceus* in intestine obtained at the treatment with doses of $2×10^9$ CFU/g. The ability of bacteria to colonize well in the digestive tract of fish caused by the *P. pentosaceus* has the ability to survive to pass the fish gut with low pH and bile salt with an alkaline pH (Maji *et al.,* 2016).

The survival rate and hematological parameters of catfish after challenge test with *Aeromonas hydrophila*

The survival rate of catfish after challenge test at the probiotics treatment (Np and Gp) was significantly different (P<0.05) compared with the treatment without probiotics (Figure 4). The Np and Gp treatment can reach the survival rate of 88.46% higher compared to K+ (53.84%) and G (65.38%). This was because the probiotics *P. pentosaceus* E2211 produces an antibacterial substance against *A. hydrophila* that was shown in inhibit zone at antagonistic test against *A. hydrophila*. Antimicrobial activity of LAB against pathogenic bacteria is due to the production of organic acids, hydrogen peroxide, and bacteriocin (Vesterlund, 2009).

Damodharan *et al.* (2015) reported that the antimicrobial activity of *P. pentosaceus* KID7 against gram-positive and gram-negative bacteria comes from organic acid production. *P. pentosaceus* has an activity of anti-*A. hydrophila* through the production of the extracellular product (ECP) that produces the maximum antagonistic activity in inhibit zone as much as 9 to 10 mm, and it can still be maintained at pH 7–9. According to Soltani *et al.* (2015), with the anti-*A. hydrophila* in *P. pentosaceus*, it helps the probiotics to pass through the digestive tract of fish and colonize dominate in the intestine of fish thus suppressing the growth of *A. hydrophila* and other various pathogens in the intestine while enhancing the immunity of fish so that it can increase the survival rate of fish higher than

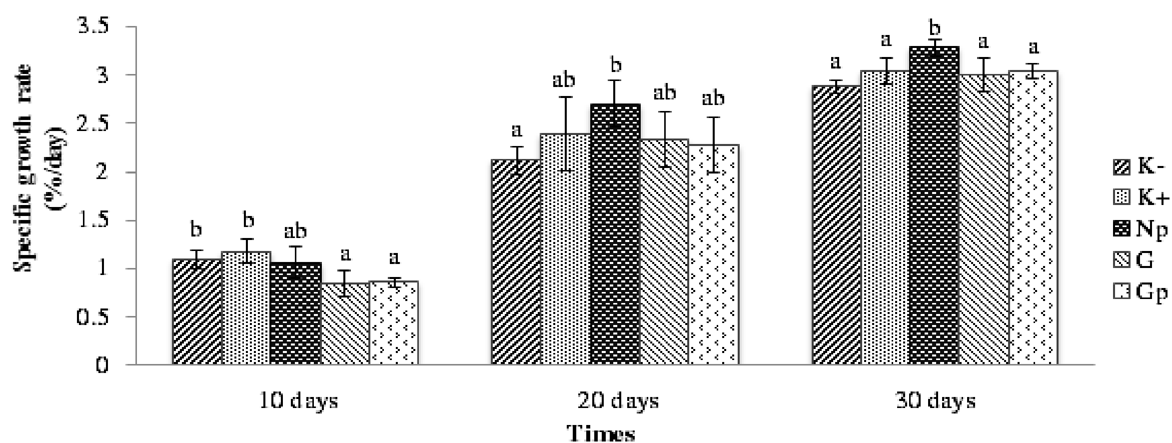

Figure 3. The specific growth rate (SGR) of catfish at 10, 20, 30 days of rearing. K- (normal catfish without addition probiotic, without challenge test), K+ (normal catfish without addition of probiotic, with challenge test), Np (normal catfish with addition of probiotic and challenge test), G (gnoto catfish without addition of probiotic, with challenge test), and Gp (gnoto catfish with addition of probiotic and challenge test). The same superscripts showed the significantly different result according to Duncan test at a confidence level of α 0.05

Table 6. The total amount of bacteria in the digestive tract of catfish after 30 days of rearing

Treatments	The total amount of intestinal bacteria (10^7 CFU/mL)	The total lactic acid of intestinal bacteria (10^6 CFU/mL)	Total probiotic (10^5 CFU/mL)
K-	7.03 ± 1.50[a]	0.42 ± 0.10[a]	0.00 ± 0.00[a]
K+	8.19 ± 0.97[a]	0.49 ± 0.05[a]	0.00 ± 0.00[a]
Np	23.38 ± 12.95[b]	59.0 ± 26.45[b]	46.5 ± 15.97[b]
G	0.03 ± 0.003[a]	0.01 ± 0.006[a]	0.00 ± 0.00[a]
Gp	1.33 ± 0.59[a]	11.7 ± 7.64[a]	7.396 ± 1.24[a]

Note: K− (normal catfish without addition probiotic, without challenge test), K+ (normal catfish without addition of probiotic, with challenge test), Np (normal catfish with addition of probiotic and challenge test), G (gnoto catfish without addition of probiotic, with challenge test), and Gp (gnoto catfish with addition of probiotic and challenge test). The numbers that followed with the same superscripts in the same column showed significantly different according to Duncan test at a confidence level of α 0.05.

the fish without probiotics.

The survival rate after challenge test was higher on catfish fed with probiotic treatment (Np and Gp) than the K+ treatment (Figure 5a, b, c, and d), it was also supported by the hematological parameters (total erythrocytes, total leukocytes, haemoglobin, and phagocytic activity after challenge test). The existence of probiotics *P. pentosaceus* E2211 can assist and accelerate the recovery process of fish by increasing the non-specific immune response and the resistance against MAS. The fish haematological parameters in this study were been in normal range of the total erythrocyte (1.3×10^6 cells/mm^3), the total leukocytes (2.15×10^4 cells/mm^3), and the haemoglobin (6.10 g/dL) (Svobodova & Vyukusova, 1991; Takashima & Hibiya 1995).

Soltani *et al.* (2015) reported that the addition of *Lactococcus lactis* probiotics (JF831150) proved to be able to increase total leukocyte, erythrocytes, and haemoglobin of Persian sturgeon (*Acipenser persicus*). The total erythrocytes and haemoglobin that was higher at the addition of probiotics treatment than K+ indicated that *P. pentosaceus* E2211 was able to maintain the fish health status, by keeping the total erythrocytes and haemoglobin in the normal range after *A. hydrophila* infection. The high amount of total leukocytes and phagocytic activity in Gp and Np treatment showed that the existence of probiotic was able to induce the production of leukocytes in a massive amount against *A. hydrophila* infection. The existence of normal microflora in association with *P. pentosaceus* E2211 in Np treatment was able to increase the immunity of fish better than *P. pentosaceus* E2211 in Gp treatment.

Sukenda *et al.* (2016), stated that the increase of total leukocytes caused by the response from the fish body against infectious diseases. Leukocyte cell is a phagocytes cell to prevent unwanted bacteria and spread the virulence factors in the fish body. The part of leukocyte

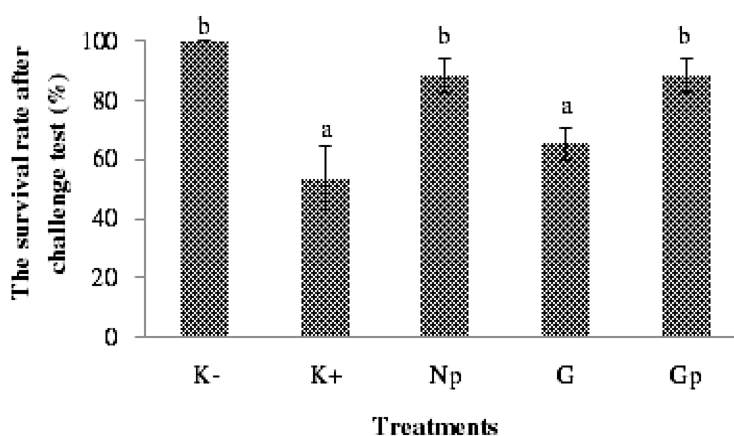

Figure 4. The survival rate of catfish after 10 days of challenge test. K- (normal catfish without addition probiotic, without challenge test), K+ (normal catfish without addition of probiotic, with challenge test), Np (normal catfish with addition of probiotic and challenge test), G (gnoto catfish without addition of probiotic, with challenge test), and Gp (gnoto catfish with addition of probiotic and challenge test). .The same superscripts showed the significantly different result according to Duncan test at a confidence level of α 0.05

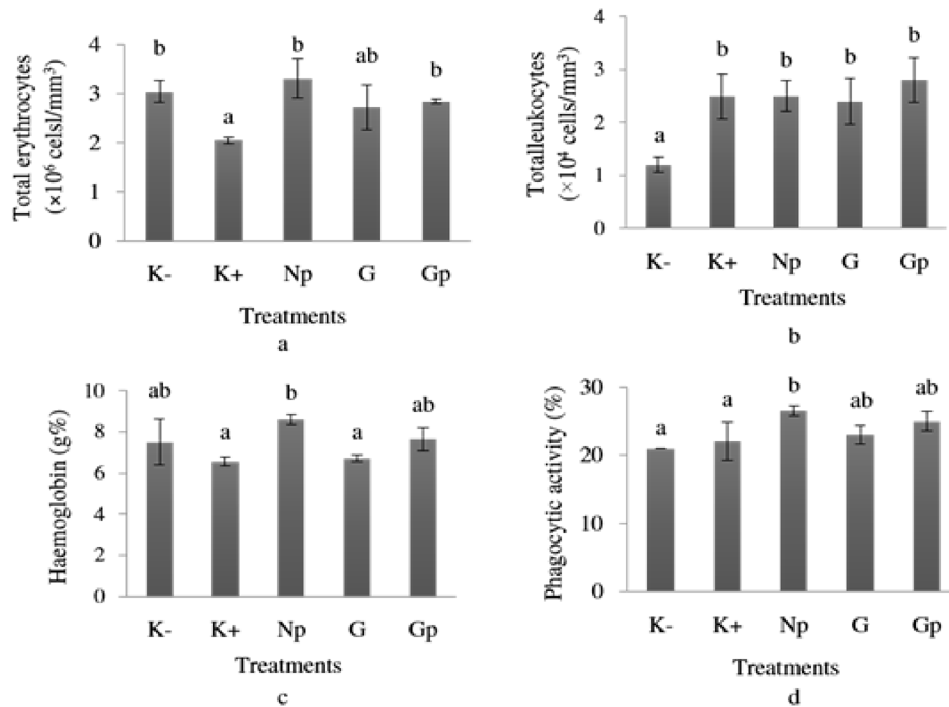

Figure 5. The hematological parameters of catfish after 10 days of challenge test: (a) The total erythrocytes; (b) The total leukocytes; (c) Haemoglobin; (d) Phagocytic activity. K– (normal catfish without addition probiotic, without challenge test), K+ (normal catfish without addition of probiotic, with challenge test), Np (normal catfish with addition of probiotic and challenge test), G (gnoto catfish without addition of probiotic, with challenge test), and Gp (gnoto catfish with addition of probiotic and challenge test). The same superscripts showed the significantly different result according to Duncan test at a confidence level of α 0.05.

cell that according to with the function are monocytes and neutrophils that indicated by the value of phagocytic activity (Utami *et al.,* 2015). Monocytes and neutrophils are the components of blood cells that generate superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxide nitrite ($ONOO^-$), hypochlorite acid (HOCl), and hydroxyl radical (OH^-) that have the high ability to kill microbes (Ellis, 2001).

CONCLUSION

The selected LAB isolates of *P. pentosaceus* E2211 is potential for probiotics candidate in normal catfish and gnotobiotic catfish (*Clarias* sp.), that indicated by the survival rate of Np and Gp treatment after challenge test, it was 88.46%, while K+ and G were only reached 53.84% and 65.38%. The performance of *P. pentosaceus* E2211 as probiotics was showed through its ability to maintaining the survival rate, the specific growth rate, the total intestinal bacteria, and the immune response of catfish to *A. hydrophila* infection. The existence of normal microflora in association with *P. pentosaceus* E2211 in Np treatment showed the best performance of probiotic with 3.28%/day of the specific growth rate, 1.78 of the feed conversion ratio, and the total intestinal bacteria reached 10^8 CFU/mL.

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