

Evaluation of single and multispecies probiotic applications for the prevention of *Motile Aeromonads Septicaemia* in gourami, *Osphronemus gourami*

Evaluasi aplikasi probiotik tunggal dan multispecies untuk pencegahan *Motile Aeromonads Septicaemia* pada ikan gurami, *Osphronemus gourami*

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ABSTRACT

The aim of this study was to evaluate single and multispecies probiotic applications for the prevention of motile aeromonads septicaemia (MAS) disease in gourami, *Osphronemus gourami*. The experiment consisted of the *in vitro* inhibition test and the *in vivo* application of probiotics in gourami. The *in vivo* assay, consisted of five treatments and five replicates, namely: negative control (K-); positive control (K+); (B) fish fed with supplementation of 1% (v/w) probiotic *Bacillus* NP5 Rif^R cells and challenged with *Aeromonas hydrophila* Rif^R; (L) fish fed with supplementation of probiotic *Lactobacillus plantarum* Cip^R 1% (v/w); challenged with *A. hydrophila* Rif^R; (BL) fish fed with mixture supplementation of *Bacillus* NP5 Rif^R 0.5% (v/w)+*L. plantarum* Cip^R 0.5%; infected with *A. hydrophila* Rif^R. Fish (29.57 ± 1.00 g) were reared in a 45 L volume aquaria with a rearing density of 10 fish/m³ for 50 days, with feeding trial three times a day. The challenge test was conducted by intramuscular injection with pathogenic *A. hydrophila* Rif^R cells (10⁶ CFU/mL) on day 41st. *In vitro* test results showed that single and multispecies probiotics significantly (P<0.05) inhibited *A. hydrophila*. *In vivo* experiment showed that probiotic supplementation treatments improved the growth performance, and microbiota diversity in the gut. The immune responses, fish resistance to *A. hydrophila* and gourami survival rate in all treatments of supplemented feed were significantly higher compared to the positive control. The best treatment, multispecies probiotics significantly (P<0.05) improved the survival of gourami 96.67% post infection with *A. hydrophila*.

Keywords: *A. hydrophila*, *Bacillus* NP5, *L. plantarum*, *O. gourami*, probiotic

ABSTRAK

Penelitian ini bertujuan untuk mengevaluasi aplikasi probiotik tunggal dan multispecies dalam pencegahan penyakit *Motile Aeromonad Septicaemia* (MAS) yang disebabkan oleh *Aeromonas hydrophila* pada ikan gurami (*Osphronemus gourami*). Penelitian terdiri atas uji penghambatan *in vitro* bakteri probiotik tunggal dan multispecies terhadap *A. hydrophila* Rif^R, dan uji *in vivo* aplikasi probiotik untuk gurami. Uji *in vivo*, terdiri dari lima perlakuan dan lima ulangan, yaitu: kontrol negatif (K-) kontrol positif (K+); (B) ikan dengan pakan suplementasi *Bacillus* NP5 Rif^R 1% dan diinfeksi *A. hydrophila* Rif^R; (L) pakan komersial dengan penambahan probiotik *L. plantarum* Cip^R 1% dan infeksi *A. hydrophila* Rif^R; (BL) ikan dengan suplementasi campuran *Bacillus* NP5 Rif^R 0.5% dan *L. plantarum* Cip^R 0.5% serta diinfeksi *A. hydrophila* Rif^R. Benih gurami (29.57 ± 1.00 g) dipelihara di dalam akuarium bervolume 45 L dengan kepadatan 10 ekor/m³ selama 50 hari, dengan pakan bersuplemen probiotik tiga kali sehari. Uji tantang dilakukan dengan menginjektikan suspensi sel patogen *A. hydrophila* Rif^R (10⁶ CFU/mL) secara *intramuscular* pada hari ke 41. Hasil uji *in vitro* menunjukkan bahwa probiotik tunggal dan multispecies dapat menghambat pertumbuhan sel *A. hydrophila* di organ hati dan ginjal secara signifikan (P<0.05). Hasil uji *in vivo* menunjukkan perlakuan aplikasi probiotik tunggal dan multispecies mampu meningkatkan kinerja pertumbuhan, keragaman mikrobiota usus. Respons imunitas, resistansi terhadap *A. hydrophila* dan kelangsungan hidup gurami di semua perlakuan pakan bersuplemen probiotik secara signifikan lebih tinggi dibandingkan kontrol positif. Perlakuan terbaik adalah probiotik multispecies meningkatkan kelangsungan hidup ikan sebesar 96.67% secara signifikan (P<0.05) pascainfeksi *A. hydrophila*.

Kata kunci: *A. hydrophila*, *Bacillus* sp. NP5, *L. plantarum*, *O. goramy*, probiotik

INTRODUCTION

Gourami, *Osphronemus gourami*, is one of the freshwater fish commodities originating from waters in Indonesia with high economic value. According to FAO (2023), Indonesia is a gourami producer and its production represents more than 98% of total world production. According to statistical data from the MMAF (2023), gourami production continues to increase every year, in 2017 it reached 45.98%. However, an increase in production that is not balanced with proper handling will be prone to disease, due to the imbalance of host, pathogen and environmental interactions. MMAF (2023), noted that there was a decrease in gourami production from 2016 to 2020 by 60.83%. This can occur due to infection with motile aeromonads septicemia (MAS) disease caused by *Aeromonas hydrophila* bacteria.

Pathogenic bacteria *A. hydrophila* is an opportunistic pathogen, which attacks hosts with low immune systems (Kuebutornye *et al.*, 2020). Infection of *A. hydrophila* has an acute mortality pattern where mortality can reach 80-100%, within a short period of death (Rozi *et al.*, 2018). This can result in high economic losses. Clinical symptoms include hemorrhage at the base of the fins, body, mouth, ulcer, fins, pale gills, protruding eyes (exophthalmia), and swelling of the liver and kidneys (Cao *et al.*, 2020). The pathogenic bacteria *A. hydrophila* produce extracellular products such as hemolysin and aerolysin which are primary virulence factors, so they can cause erythrocyte hemolysis in fish (Kuebutornye *et al.*, 2020).

Several journals recorded the mortality of gourami caused by *A. hydrophila* in Indonesia. The outbreaks located in Pare, Kediri, East Java it was reported that the mortality reached 70% of the fish with average weight of 35 g each (Satyantini *et al.*, 2016). Whereas the gourami mortality in Blitar and Bantul districts, was reported approximately reached up to 70% of the population with average individual weight of 400 g (Rozi *et al.*, 2018). The largest mortality was reported occurred in Banyumas, where gourami broodstocks were infected by *A. hydrophila* with a mortality percentage reached up to 90% of the total population (Khumaidi & Hidayat, 2018). The most common way to overcome disease outbreaks is with the use of antibiotics (Rozi *et al.*, 2018).

However, the use of antibiotics can cause bacteria to be resistant to antibiotics,

environmental damage, bioaccumulation, and food safety issues (Puvanendran *et al.*, 2021). Along with the increasing restrictions on the use of antimicrobials, an alternative that can be applied is the use of probiotic bacteria as an effort to control *A. hydrophila*. Probiotics are defined as live microorganisms that, when applied in sufficient quantities, provide health and benefit to the host (Bahaddad *et al.*, 2022). Probiotic strains may not necessarily be hostile to pathogenic bacteria, but may indirectly prevent cellular or tissue damage to the host caused by some potential pathogenic mechanisms. Evaluation of the effectiveness of probiotic applications using single or multi-species probiotics has been demonstrated to improve growth performance, to increase the disease resistance, and to stimulate the immune system (Kuebutornye *et al.*, 2020).

In this study, the bacteria used were *Bacillus* NP5, *Lactobacillus plantarum*, and a combination of both. The single bacterium *Bacillus* NP5 is able to produce amylase, protease, and lipase enzymes in the process of food digestion, thus facilitating intestinal performance. *Bacillus* sp. can contribute to increase the feed utilization efficiency, nutrient absorption, and growth rate (Bahaddad *et al.*, 2022). Another benefit that *Bacillus* sp. bacteria can provide is by producing lysozyme which can destroy the cell walls of pathogenic bacteria, as well as producing the enzyme catalase, where the catalase enzyme can convert hydrogen peroxide (H_2O_2) compounds into simpler compounds, namely air (H_2O) and oxygen (O_2) (Simon *et al.*, 2021).

Lactobacillus plantarum has capability in producing amylase, protease, and lipase enzymes as growth promoters (Sulistiani, 2017). *L. plantarum* bacteria can also produce lactic acid which modulates the immune responses, and produces plantaricin as an antimicrobial compound that can kill bacteria by disrupting the protein synthesis of pathogenic bacteria (Meiyasa *et al.*, 2017). The demonstrations of many beneficial aspects produced by probiotic bacteria of *Bacillus* and *L. plantarum* and further combination of mixed cultures of both are expected to synergize with each other. *Bacillus* NP5 bacteria can produce catalase enzymes that can hydrolyze H_2O_2 compounds produced by *L. plantarum* into H_2O as an acid neutralizer in the body so as to reduce the effects of oxidative stress and O_2 as cell respiration so as to modulate immune responses.

The work of lysozyme from *Bacillus* in damaging the cell wall of pathogenic bacteria can also help plantaricin to enter pathogenic cells and inhibit protein synthesis of pathogenic bacteria (Kuebutornye *et al.*, 2020). Based on these effectiveness and benefits, further research is needed to evaluate the application of single and multi-species probiotics in an effort to prevent MAS disease in gourami (*O. gourami*). Several studies related to the addition of probiotics, (Puvanendran *et al.*, 2021) the addition of probiotic *Carnobacterium divergens* increased growth parameters and disease resistance in cod larvae (*Gadus morhua*), with a survival rate of 63.3%. Research by Beck *et al.* (2015), the addition of *Lactococcus lactis* and *L. plantarum* to *Olive flounder*, found anti-inflammatory encoding genes and pro-inflammatory encoding genes that can synergize in inhibiting pathogen growth and improving growth performance with a 55% survival rate.

Research by Kuebutornye *et al.* (2020), a combination of probiotic bacteria *B. velezensis*, *B. subtilis*, *B. amyloliquefaciens* after being infected with *A. hydrophila* reduced mortality by 30% and can increase the mucosal immune system, and improve the gastrointestinal tract. According to Kong *et al.* (2020) feed added with *Lactococcus lactis* and *Enterococcus faecalis* to *Channa argus* fish can improve growth parameters, humoral immune response, and the survival rate reaches 63.3%. According to Chen *et al.* (2020), feed supplemented with *Bacillus* spp. in *Ctenopharyngodon idella* fish can improve growth performance, immunity, antioxidant function, response to hypoxic stress and resistance to *A. hydrophila*. Based on the effectiveness and benefits, further research is needed to evaluate the application of single and multi-species probiotics in preventing MAS disease in gourami (*O. goramy*).

MATERIALS AND METHODS

Preparation of probiotic bacteria

The probiotic bacteria used in this study were *Bacillus* NP5 from the Aquatic Organism Health Laboratory, Department of Aquaculture, FPIK IPB and *L. plantarum* from the Food and Nutrition Culture Collection Laboratory, Gadjah Mada University. Both probiotic bacteria were marked with rifampicin and ciprofloxacin antibiotic resistance (*Bacillus* NP5 Rif^R and *L. plantarum* Cip^R), respectively. *Bacillus* NP5 Rif^R and *L.*

plantarum Cip^R were cultured on Luria Bertani broth (LB) and incubated on a waterbath shaker (140 rpm, 29°C), *Bacillus* NP5 Rif^R culture for 12 h and *L. plantarum* Cip^R for 24 h. Both freshly harvested isolates were then centrifuged (3000 rpm) for five min to obtain probiotic pellets. The probiotic pellets were homogenized in 0.1 mL of sterile phosphate-buffered saline (PBS) Widanarni *et al.* (2019).

Research design

This research consists of two stages, *in vitro* tests in the form of co-culture tests and *in vivo* tests, the application of probiotic bacteria to goramy. The co-culture test aims to determine the ability of probiotic bacteria *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R, and to determine the ability of multispecies bacteria to synergize in inhibiting the growth of *A. hydrophila* Rif^R. This treatment uses the scatter disk method on selective RS (rhimler shotts) medium which has been added with rifampicin antibiotic as a marker. This study consisted of four treatments with three repetitions, namely: (A) culture of *A. hydrophila* Rif^R as a positive control; (BA) co-culture of *Bacillus* NP5 Rif^R with *A. hydrophila* Rif^R; (LA) co-culture of *L. plantarum* Cip^R with *A. hydrophila* Rif^R bacteria; (BLA) co-culture of *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R with *A. hydrophila* Rif^R bacteria. Then the results of the joint culture were compared with the positive control.

If the growth of *A. hydrophila* Rif^R colonies in positive control treatment was more abundant compared with the results of co-culture, showed that probiotic bacteria can inhibit the growth of pathogenic bacteria (Widanarni *et al.*, 2019). *In vivo* test, this study consisted of five treatments and five replicates, namely: (K-) fish fed with the commercial feed without probiotics and injected with PBS; (K+) fish fed with commercial feed without probiotics, but challenged with *A. hydrophila* Rif^R; (B) fish fed with commercial feed supplemented with probiotic *Bacillus* NP5 Rif^R 1% and infected with cell suspension of *A. hydrophila* Rif^R; (L+) fish fed with commercial feed supplemented with probiotic *Bacillus* NP5 Rif^R 1% and infected with bacteria *A. hydrophila* Rif^R; (L) commercial feed given with the addition of probiotic *L. plantarum* Cip^R 1% and infection with *A. hydrophila* Rif^R; (BL) commercial feed given with the addition of *Bacillus* NP5 Rif^R 0.5% and *L. plantarum* Cip^R 0.5% and infection with *A. hydrophila* Rif^R.

Preparation of test feed

The feed used was commercial feed with a protein content of 35%. In the preparation of probiotic bacteria, the cells were cultured in liquid Luria Bertani (LB) medium then incubated and shaken at room temperature for 12 hours (for *Bacillus* NP5) and 24 hours (for *L. plantarum*). After the cell harvest by centrifugation, the pellet was rinsed using phosphate buffer saline (PBS) solution three times. The bacterial cell suspension was diluted to cells density of 10^8 CFU/mL.

The probiotic cell was added with the sterile progol and 1% (v/w) PBS solution as a binder. Probiotic cell suspension were homogenized using sterile screw tubes accordingly to each treatment. The cell suspension was then homogenized using vortex mixer, evenly sprayed on the pellet feed using a syringe and then dried. The supplemented feed was then tested for probiotic cells viability by cell counting using the total plate count (TPC) method. TPC was conducted by spread plating on LB agar medium that had been added 50 μ g/mL rifampicin (for *Bacillus* NP5 Rif^R) and with 50 μ g/mL ciprofloxacin (for *L. plantarum* Cip^R).

The cells viability in feed was approximately at concentration of 10^6 CFU/g of dried pellet, for each species. In the multispecies probiotic treatment, the cells were diluted to a density of 10^8 CFU/mL. The dose used in the probiotic treatment was 0.5% (v/w) for each probiotic bacteria. The cells viability of probiotic was counted using the TPC method by spread plated on media containing antibiotics accordingly the cells marker. The viability of the TPC results was approximately to cells density of 10^6 CFU/g of dried feed.

Preparation of rearing media

The tanks used for the experiment were 15 units glass aquaria with dimensions of 50×30×30 cm³. Each tank was filled with freshwater at height level of 30 cm, each equipped with filtration and aeration. The gourami fish used was originating from the gourami fish farmers in Bogor, West Java. Fish with an initial weight of 29.57 ± 1.00 g were acclimatized for one week and fed with standard commercial feed during the acclimatization process.

The fish were confirmed to be free of *A. hydrophila* infection, using the PCR method of Persson *et al.* (2015), the primers used in PCR were A-hyd *gyr-B* F(533) 5'-AGTCTGCCGCCAGTGGC-3' and A-hyd R(677) 5'-CRCCATCGCCTGTTCG-3' with

a target size of 144 bp, specifically targeting *A. hydrophila*. After the acclimatization process was completed, the fish were reared at fish density of 60 fish/m² or 10 fish/container. Rearing period for feed supplementation was carried out for 50 days and the experimental fish were fed three times a day at 08.00, 13.00, and 18.00 at local time, afterwards the challenge test with pathogenic cells was performed.

Challenge test

The challenge test was conducted on day 50 of probiotic application. Each fish (except the negative control (K-)) were injected intramuscularly with a cells suspension of *A. hydrophila* Rif^R as much as 0.1 ml 10^6 CFU/mL. The fish of the negative control treatment (K-) was injected with PBS. After the challenge test, the fish were continuously reared for 14 days and fed with standard commercial feed.

Test parameters

Growth performance

After 50 days of probiotic application, fish specific growth rate (SGR), feed conversion ratio (FCR) and survival rate (SR) by Simon *et al.* (2020).

Bacterial cells monitoring in the gut

The intestinal probiotic population was counted using the TPC method after 50 d of feed supplementation. The gourami sample intestine was taken as much as 0.1 g, then grinded and serially diluted using sterile PBS solution. The diluted samples were spread plated on LB agar media, LB agar media + 50 μ g/mL rifampicin (for *Bacillus* NP5 Rif^R) and LB agar media + 50 μ g/mL ciprofloxacin (for bacteria *L. plantarum* Cip^R) to determine the *Bacillus* NP5 Rif^R cells population and *L. plantarum* Cip^R in the gourami digestive tract in each treatment. Total bacterial colonies were counted and presented in colony forming units (CFU/g) referring to the procedure of Widanarni *et al.* (2019).

Enzymatic activity

Measurement of digestive enzyme activity was carried out at the end of the feed application treatment, namely on day 50 at the Animal Nutrition Laboratory, Faculty of Animal Husbandry, IPB. The measured digestive enzyme activities of goramy include amylase, protease, and lipase enzyme activities. Digestive tract sampling. The digestive tract of vaname shrimp

was taken aseptically and put into a sample bottle as much as 0.1 g. Then the samples are stored in a deep freezer at -80°C until ready for testing.

Fish immune parameters

Calculation of total erythrocytes, total hemoglobin, total erythrocytes, phagocytic activity observed through blood slides and respiratory burst activity refers to the procedure of Witeska *et al.* (2022). Total leukocytes, and lysozyme activity refer to the procedure of Seibel *et al.* (2021).

Cells population of *A. hydrophila* in target organs

The total number of pathogenic *A. hydrophila* in the target organ was calculated using the TPC method. A total of 0.1 g of target organ samples (kidney and liver) were homogenized in 0.9 mL of sterile PBS with serial dilution technique. Then 25 μL of each dilution was spread plated on petri dish containing RS medium containing rifampicin. Total counting of *A. hydrophila* pathogenic bacteria in the target organ was carried out on day 54, 57, 60 and day 63 referring to the procedure of Widanarni *et al.* (2019).

Fish resistance to *A. hydrophila*

Fish resistance was measured by calculating the survival of gourami that have been tested with *A. hydrophila* Rif^R. The survival rate value of the fish was counted by procedure according to Chen *et al.* (2020).

Histopathology analysis

The histopathology was carried out with fish samples especially from liver and kidney samples. Samples were taken from control treatments and

fish with supplemented feed treatments, as much as 1×1 cm. Organ samples were fixed using Buffered Neutral Formalin (BNF) solution for 24–72 h for histopathology preparation. After that, the process of dehydration, clearing, embedding, paraffin blocking, sectioning was carried out with a thickness of 3–5 mm and staining was continued. The stain used was hematoxylin and eosin. The slices were observed under a microscope with 40 times magnification.

RESULTS AND DISCUSSION

Result

In vitro test

The results of the *in vitro* co-culture test of probiotic bacteria and pathogenic bacteria are presented in Figure 1. The co-culture results showed that treatment A (8.35 ± 0.28 log CFU/mL) as a positive control (A), was higher ($P < 0.05$) than treatments BA (2.70 ± 0.36 log CFU/mL), LA (2.46 ± 0.18 log CFU/mL), and BLA (1.71 ± 0.56 log CFU/mL). This proves that single probiotic bacteria can inhibit the growth of *A. hydrophila* Rif^R, and multi-species probiotic bacteria *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R can synergize in killing *A. hydrophila* bacteria.

Confirmation of gourami free of *A. hydrophila*

The confirmation of pathogen-free gourami fish was carried out using the PCR method, the results are presented in Figure 1. The target size of *A. hydrophila* is indicated by the appearance of a 144 bp band in the positive control. The gourami blood samples (1–7) showed no band, indicating that the gourami were free of *A. hydrophila* and could be used for research.

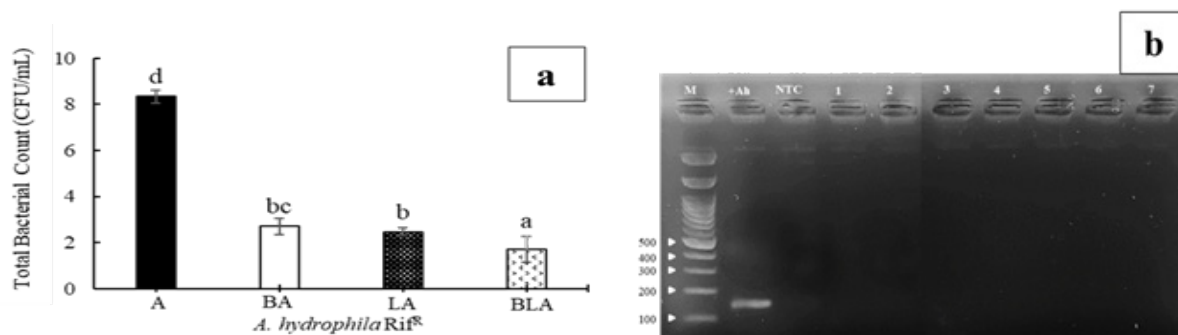


Figure 1. (a-b). (a) Co-culture test of probiotic bacteria and pathogenic bacteria; (b) confirmation of *A. hydrophila*-free gourami. Different letters above the bars indicate significant differences ($P < 0.05$). Positive control (A), *Bacillus* NP5 Rif^R + *A. hydrophila* Rif^R co-culture (BA); *L. plantarum* Cip^R + *A. hydrophila* Rif^R co-culture (LA); *Bacillus* NP5 Rif^R + *L. plantarum* Cip^R + *A. hydrophila* Rif^R co-culture (BLA). Marker DNA ladder (M), *A. hydrophila* positive control (+Ah), no template control (NTC), gourami blood samples (1-7).

Growth performance

The final biomass (Bt), survival rate (SR), specific growth rate (SGR), and feed conversion ratio (FCR) of gourami after being treated for 50 days are presented in Table 1. The initial biomass (B₀) of gourami was not significantly different (P<0.05) in all treatments, while the final biomass (B_t) of the BL treatment was significantly higher (P<0.05) compared to the other treatments. Specific growth rate in the BL treatment showed significantly higher results (P<0.05) than the other treatments. Feed conversion ratio in the BL treatment showed a significantly lower value (P<0.05) than the other treatments. The application of probiotics in feed for 50 days can increase SGR which results in a decrease in FCR value, so that it can increase the final weight and TKH of gourami at the end of experiment.

Total probiotic bacteria in the digestive tract

After 50 days of probiotic application, total bacteria and total probiotic bacteria in the digestive tract are presented in Table 1. Calculation of total bacterial colonies in the digestive tract in treatments B, L, and BL showed significantly higher results (P<0.05) than the control treatment. Total colonies of probiotic bacteria *Bacillus* NP5 Rif^R were only found in treatments B and BL. Probiotic *L. plantarum* Cip^R was only found in the L and BL treatments. In the *Bacillus* NP5 Rif^R

and control treatments, no probiotic *L. plantarum* Cip^R was found, while in the *L. plantarum* Cip^R and control treatments, no probiotic *Bacillus* NP5 Rif^R was found.

Digestive enzyme activity

After 50 days of probiotic application, enzyme activities in the digestive tract are presented in Table 1. Enzyme activities in the digestive tract in treatments B, L, and BL showed significantly higher results (P<0.05) than the control treatment. Amylase enzyme activity in the BL treatment showed significantly higher results (P<0.05) than the control treatment. Protease enzyme activity in the BL treatment showed significantly higher results (P<0.05) than the control treatment. Lipase enzyme activity in the BL treatment showed significantly higher results (P<0.05) than the control treatment (0.12 ± 0.00 IU/mL).

Immune response

The application of *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R bacteria in feed affects the survival rate (TKH) and hematological profile of gourami after *A. hydrophila* Rif^R infection Figure 2. Total erythrocytes (TE), hematocrit (Hc), hemoglobin (Hb), total leucocytes (TL), phagocytic activity (PA), respiratory burst activity (RB), and lysozyme activity (LA) have different values in each treatment, which represents changes

Table 1. Initial biomass (B₀), final biomass (B_t), specific growth rate (SGR), feed conversion ratio (FCR), survival growth rate (SGR), total digestive tract bacteria (TB), *Bacillus* NP5 Rif^R in the digestive tract and *L. plantarum* Cip^R in the digestive tract, in gourami after 50 days of probiotic bacteria application.

Growth Parameter	Treatment				
	K(-)	K(+)	B	L	BL
B ₀ (g)	28.90 ± 1.25 ^a	29.57 ± 1.00 ^a	29.05 ± 1.52 ^a	28.73 ± 0.48 ^a	29.21 ± 1.36 ^a
B _t (g)	72.52 ± 0.77 ^{ab}	70.93 ± 0.45 ^a	82.51 ± 2.27 ^b	79.95 ± 2.52 ^b	92.89 ± 0.85 ^c
SGR (%/day)	1.55 ± 0.06 ^a	1.47 ± 0.05 ^a	1.76 ± 0.04 ^b	1.72 ± 0.06 ^b	1.95 ± 0.06 ^c
FCR	2.15 ± 0.10 ^c	2.20 ± 0.10 ^{cd}	1.79 ± 0.13 ^b	1.84 ± 0.05 ^b	1.61 ± 0.03 ^a
SR (%)	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a
TB (Log CFU/g)	8.62 ± 0.21 ^b	8.68 ± 0.08 ^b	7.92 ± 0.41 ^a	8.54 ± 0.38 ^b	9.86 ± 0.06 ^c
<i>Bacillus</i> NP5 Rif ^R (Log CFU/g)	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	7.93 ± 0.40 ^b	0.00 ± 0.00 ^a	8.48 ± 0.29 ^c
<i>L. plantarum</i> Cip ^R (Log CFU/g)	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	7.87 ± 0.20 ^b	8.64 ± 0.39 ^c
Amylase (IU/mL)	2.41 ± 0.02 ^a	1.80 ± 0.01 ^b	2.51 ± 0.01 ^c	3.15 ± 0.02 ^d	3.58 ± 0.02 ^e
Protease (IU/mL)	0.22 ± 0.00 ^a	0.25 ± 0.00 ^b	0.30 ± 0.00 ^c	0.29 ± 0.00 ^c	0.34 ± 0.00 ^d
Lipase (IU/mL)	0.12 ± 0.00 ^a	0.12 ± 0.00 ^a	0.14 ± 0.00 ^b	0.12 ± 0.00 ^a	0.15 ± 0.00 ^c

Notes: Different superscript letters in the same row indicate significant differences (LSD, P<0.05). Values presented are mean and standard deviation. Positive control (K+), negative control (K-), *Bacillus* NP5 Rif^R (B), *L. plantarum* Cip^R (L), *Bacillus* NP5 Rif^R + *L. plantarum* Cip^R (BL).

in the health status of gourami. Total gourami erythrocytes on day 0 showed different values in treatment B ($1.59 \pm 0.19 \times 10^6$ cells/mm³) which was significantly higher ($P < 0.05$) than treatment L, BL and control. Increased physical activity and metabolism of fish, balanced nutrient availability, optimal aquatic environment can meet their oxygen and metabolic needs can indirectly affect the increase in total erythrocytes.

Total gourami erythrocytes after 50 days of probiotic application, BL treatment ($3.66 \pm 0.25 \times 10^6$ cells/mm³) showed a significantly higher increase ($P < 0.05$) than the other treatments. On day 54 post-infection, there was a decrease in TE in all treatments, and at the end of the observation (day 63) the BL treatment ($3.44 \pm 0.27 \times 10^6$ cells/mm³) showed significantly higher TE results ($P < 0.05$) than the other treatments. Haematocrit levels of gourami on day 0 showed values that were not different ($P < 0.05$) in all treatments $18.76 \pm 0.86\%$. After 50 days of probiotic application, the BL treatment ($39.31 \pm 0.28\%$) showed a significantly higher ($P < 0.05$) than other treatments. On day 54 post-infection, there was a decrease in Hc in all treatments, and at the end of observation (day 63) the BL treatment ($39.38 \pm 0.65\%$) showed higher Hc results ($P < 0.05$) compared to other treatments.

Haemoglobin levels of gourami on day 0 showed different values with treatment B (8.37 ± 0.25 g/%) significantly higher ($P < 0.05$) than treatment L, BL and control. Hemoglobin levels of gourami after 50 days of probiotic application, the BL treatment (14.67 ± 0.23 g/%) showed a significantly higher increase ($P < 0.05$) than the other treatments. On day 54 post-infection, there was a decrease in Hb in all treatments, and at the end of observation (day 63) the BL treatment (14.40 ± 0.32 g/%) showed higher Hb results ($P < 0.05$) compared to other treatments.

Total leucocyte after 50 days of probiotic application, the BL treatment ($4.39 \pm 0.47 \times 10^4$ cells/mm³) showed a significantly higher increase ($P < 0.05$) than the other treatments. On day 54 post-infection, there was an increase in TL in all treatments, and on day 57 there was a decrease in TL values in all treatments except the K(+) treatment. The 63rd day showed the results of TL returning to normal, with the TL value in the BL treatment ($3.506 \pm 0.18 \times 10^4$ cells/mm³) significantly higher ($P < 0.05$) than the other treatments.

The phagocytic activity of gourami at the beginning of study *Bacillus* showed a value that

was not different ($P < 0.05$) in all treatments $9.67 \pm 0.58\%$. The phagocytic activity of gourami after 50 days of probiotic application, BL treatment ($30.31 \pm 0.26\%$) showed a significantly higher increase ($P < 0.05$) than the other treatments. On day 54 post-infection, there was an increase in FA in all treatments, and on day 57 there was a decrease in FA values in all treatments except the K(+) treatment. Day 63 showed the FA results returned to normal, with the FA value in treatment L ($32.07 \pm 0.18\%$) significantly higher ($P < 0.05$) than the other treatments.

The lysozyme activity of gourami on day 0 showed a value that was not significantly different ($P > 0.05$) ranging from 230.60 ± 19.16 U/mL. The lysozyme activity of gourami after 50 days of probiotic application, BL treatment (0.86 ± 0.10) showed a significantly higher increase ($P < 0.05$) than other treatments. On day 54 post-infection, there was an increase in LA in all treatments, and on day 57 there was a decrease in LA values in all treatments except the K (+) treatment. Day 63 showed the LA results returned to normal, with the LA value in treatment L (0.86 ± 0.04 U/mL) significantly higher ($P < 0.05$) than the other treatments.

The respiratory burst activity of gourami on day 0 showed a value not significantly different ($P > 0.05$) ranging from 0.14 ± 0.03 . Lysozyme activity of gourami after 50 days of probiotic application, BL treatment (915.00 ± 68.74 U/mL) showed a significantly higher increase ($P < 0.05$) than other treatments. On day 54 post-infection, there was an increase in LA in all treatments, and on day 57 there was a decrease in LA values in all treatments except the K(+) treatment. On the 63rd day, the results of LA returned to normal, with the LA value in the BL treatment (845.00 ± 31.22 U/mL) significantly higher ($P < 0.05$) than the other treatments.

On the 63rd day post-infection, the SR values of treatments B ($83.33 \pm 5.77\%$), L ($83.67 \pm 5.77\%$), and BL ($97.67 \pm 5.77\%$) were higher than K(+) ($43 \pm 5.77\%$) ($P < 0.05$). The survival rate of gourami after *A. hydrophila* infection, in treatments B, L, and BL ($p > 0.05$ higher) was significantly different compared to the positive control (Appendix 13). The highest survival rate was found in the BL treatment, amounting to $96.67 \pm 5.77\%$, not significantly different from the negative control. It is proven that the application of single and multi-species probiotics can increase the resistance of gourami to *A. hydrophila* infection.

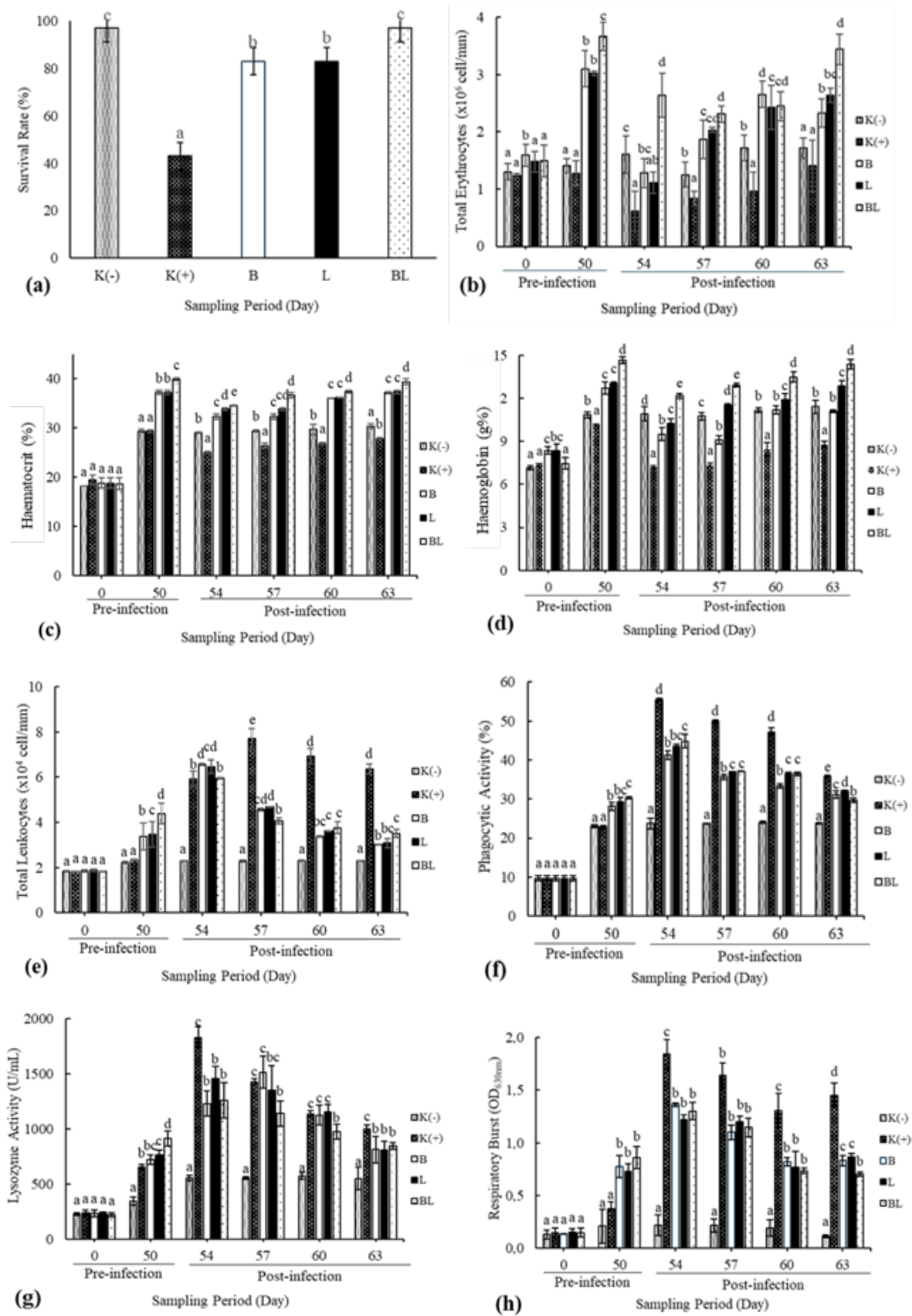


Figure 2. Survival rate dan hematological parameters of goramy: (a) Survival rate; (b) total erythrocytes; (c) hematocrit; (d) hemoglobin; (e) total leukocytes; (f) phagocytic activity; (g) respiratory burst activity; (h) lysozyme activity. Different letters above the bars indicate significant differences (LSD, P<0.05). The values presented are the mean and standard deviation. Positive control (K+), negative control (K-), *Bacillus* NP5 Rif^R (B), *L. plantarum* Cip^R(L), *Bacillus* NP5 Rif^R + *L. plantarum* Cip^R (BL).

Population of A. hydrophila cells in the target organs

The population of *A. hydrophila* Rif^R in the target organs of post-infected gourami in the liver and kidney is presented in Figures 3a and 3b below. The total pathogenic bacteria of *A. hydrophila* in the liver decreased from day 63 except for the positive control treatment. The BL treatment (0.12 ± 0.02 log CFU/g) showed significantly lower values ($P < 0.05$) compared to the other treatments. Total pathogenic bacteria *A. hydrophila* Rif^R in the kidney organ showed a decrease until day 63 except the positive control treatment. The BL treatment (0.11 ± 0.01 log CFU/g) showed a significantly lower value ($P < 0.05$) compared to the other treatments.

Histopathology

The histopathology of liver and kidney tissues of gourami infected with *A. hydrophila*, compared to the positive control and negative control is presented in Figure 4. Histopathological results showed that there were changes and differences that appeared visually in the liver and kidney tissues of control and treated fish. The K(-) treatment did not show any damage, while the K(+) treatment and probiotic application showed that the organs were damaged by *A. hydrophila* infection. The liver and kidney organs experienced tissue necrosis. Kidney organ damage is indicated by the presence of haemorrhage and inflammation, as a reaction of immune cells in response to foreign substances that enter the body. Liver damage in fish was found to have granulomas, vacuolisation, and congestion.

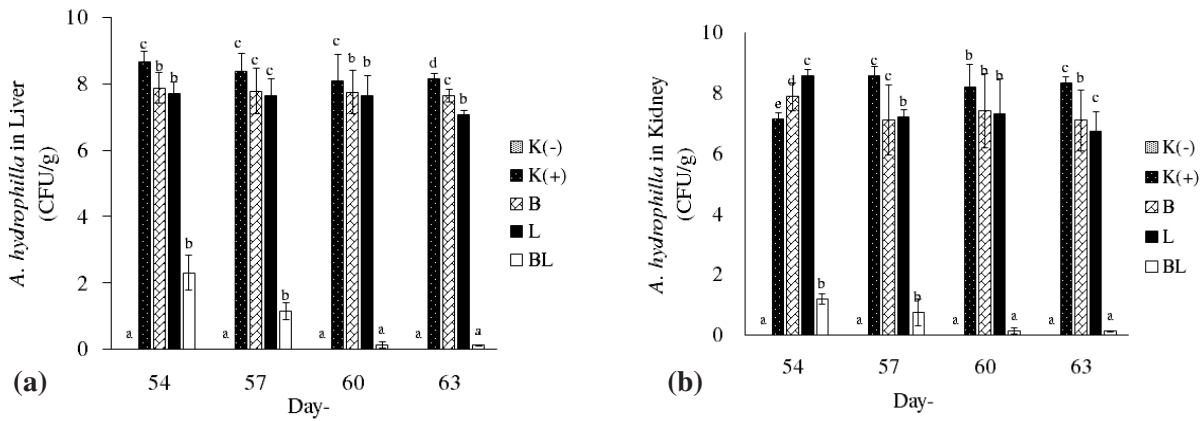


Figure 3. (a-b). Number of *A. hydrophila* in target organs: (a) liver (b) kidney of gourami. Different letters above the bars indicate significant differences (LSD, $P < 0.05$). Values presented are mean and standard deviation. Positive control (K+), negative control (K-), *Bacillus* NP5 Rif^R (B), *L. plantarum* Cip^R (L), *Bacillus* NP5 Rif^R + *L. plantarum* Cip^R (BL).

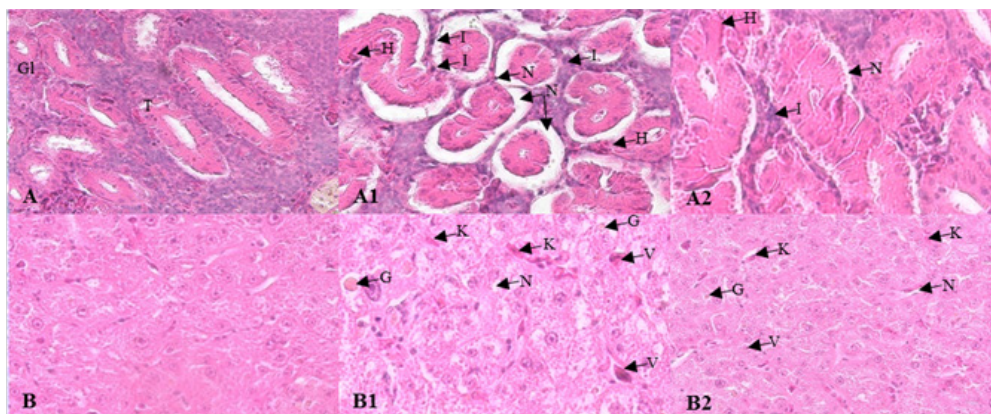


Figure 4. Histopathology of liver and kidney tissue in gourami. Controls showing kidney tissue with normal glomeruli (Gl) and renal tubules (T) (A); positive control of kidney tissue infected with *A. hydrophila* (A1); probiotic application treatment of kidney tissue infected with *A. hydrophila* (A2). Control showing liver tissue with normal sinusoids (S) (B); positive control of liver tissue infected with *A. hydrophila* (B1); probiotic application treatment of liver tissue infected with *A. hydrophila* (B2). Staining using haematoxylin and eosin (H&E) method with 40× magnification. Damage is indicated by black arrows, necrosis (N), congestion (K), granuloma (G), vacuolation (V), hemorrhagic (H), inflammation (I).

Discussion

Application of single or multi-species probiotic bacteria can have different effects on the host. Initial testing was carried out *in vitro*, probiotic bacteria were cultured simultaneously with pathogenic bacteria. The results of co-culture showed that probiotic bacteria were able to inhibit the growth of the pathogen *A. hydrophila* Rif^R (Figure 1a). This can be seen from the total colonies of *A. hydrophila* Rif^R bacteria in rifampicin RS media, treatment A was significantly higher ($P < 0.05$) compared to treatments BA, LA, and BLA.

This proves that the multispecies probiotic bacteria *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R can work together in inhibiting the pathogenic bacteria *A. hydrophila* Rif^R. This is indicated by the number of *A. hydrophila* Rif^R colonies that grow in cultures added with probiotic candidate bacteria is lower than the other treatments. This is in line with the opinion Destianingrum *et al.* (2017), that pathogenic bacteria cultured simultaneously with probiotic candidate bacteria show a lower number of pathogenic bacterial colonies compared to the control. This is also supported by the opinion of Simon *et al.* (2020), inhibition of bacterial growth can occur due to the production of antimicrobial compounds and competition for available energy sources.

This can illustrate how different bacterial populations occupy the same ecosystem. Confirmation of the health status of gourami before they are used for research is necessary to avoid biasing the results (Figure 1b). The health status of the test animals has been confirmed to be free of pathogenic bacteria *A. hydrophila* and can be used in research. This is supported by Beck *et al.* (2015) which states that fish that have been infected with pathogenic bacteria will more easily recover their bodies, because the host has stored genetic information from the type of pathogen that infects (bacteria, viruses, or fungi).

The synergistic ability of probiotic bacteria *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R was analyzed in a co-culture tests. Furthermore, an *in vivo* experiment was carried out to determine the ability of probiotic bacteria applied through feed in improving the growth performance and immune response of gourami for 50 days. The results of the application of probiotic bacteria in feed for 50 days of treatment, showed that the total colony of probiotic bacteria was higher than the control treatment. This indicates that probiotic bacteria are able to survive in the gut of gourami,

data is shown in Table 1.

Any type of bacteria added to the gut must be able to compete with other pre-attached microflora in order to survive. Probiotic strains must be able to attach to the intestinal mucus layer and use mucus as a source of nutrients to colonise, have persistent properties and be able to proliferate in the digestive tract of fish (Djauhari *et al.*, 2016). The ability to attach *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R bacteria to the gourami intestine, affects the increase in amylase, protease and lipase enzyme activities in the gourami intestine (Table 1).

This is in line with the opinion of Chen *et al.* (2020) that the use of *Bacillus* spp. bacteria affects the activity of digestive enzymes, because probiotic bacteria can produce digestive enzymes, including protease, lipase, amylase, and cellulase which are the main enzymes in contributing to improving the function of nutrient absorption in the digestive tract (intestine) so as to increase growth and feed utilisation. In the study of Zhao *et al.* (2019) the addition of feed using *B. pumilus* can increase host nutrient utilisation through the production of digestive enzymes and help the host improve the balance of its gut microbiota. Meanwhile, according to Tamilarasu *et al.* (2019) the increase in digestive enzyme activity in probiotic treatments is due to the production of exogenous digestive enzymes by probiotic bacterial species, after successfully colonizing the intestinal tract, which then induces the production of endogenous digestive enzymes.

Increased enzyme activity can indirectly improve growth performance in gourami, the results can be seen in (Table 1). Increased growth performance in treatments B, L, and BL proved to be able to increase higher growth performance compared to the control, with the best results in the BL treatment. Bacterial enzymatic hydrolysis can stimulate an increase in SGR by reducing the FCR value, increasing body weight and survival rate of gourami until the end of experiment. The increase in SGR may be associated with an increase in protease activity detected in the gut.

According to Akter *et al.* (2019) Gram-positive bacteria, especially *Lactobacillus* sp. strains, have the ability to produce exogenous enzymes, exogenous enzymes secreted by probiotic cells can help synthesize the endogenous enzymes secreted by the fish body. Compared with endogenous enzymes, these exogenous enzymes are able to tolerate fluctuating pH, so that nutrient utilisation can be fulfilled. Probiotic bacteria *Bacillus*

NP5 Rif[®] and *L. plantarum* Cip[®] can increase amylase and lipase enzymes that can trigger the production of essential fatty acids by lipid digestion and assimilation of these essential fatty acids, resulting in higher growth in fish. This is reinforced by the opinion of Lin *et al.* (2019) that efficient lipid utilisation from feed supplemented with *L. plantarum* can increase lipase activity in muscle tissue and peritoneal cavity in tilapia.

Physiological responses of normal and disease-infected fish can be detected by measuring hematological profiles. Based on the results of hematological measurements in Figure 2, total erythrocytes, hematocrit, and hemoglobin levels increased after 50 days of probiotic application. The physiological response of normal fish and fish infected with disease can be detected by measuring the hematological profile. Based on the results of hematological measurements, total erythrocytes, hematocrit, and hemoglobin levels increased after 50 days of probiotic application.

However, on the 54th day after infection, all treatments decreased, this was due to the fact that *A. hydrophila* bacteria can perform quorum sensing and can produce extracellular products in the form of α and β aero-hemolysin which can cause the lysis of red blood cells, thus reducing total erythrocytes. The decrease in erythrocytes also affects hematocrit and hemoglobin levels because the three parameters are interrelated. Hematocrit is the percentage of the volume of red blood cells (erythrocytes) in the blood. Hemoglobin is a protein in red blood cells (erythrocytes) that has the function of transporting oxygen from the lungs to the rest of the body and transporting carbon dioxide back to the lungs.

This is supported by the opinion of Djauhari *et al.* (2016) *A. hydrophila* can produce extracellular products (α and β hemolysin, aerolysin, enterotoxins ACT, ALT and AST, protease and Rnase) which can cause necrotic erythrocytes, erythrocyte hemolysis and iron ions, resulting in a decrease in the number of red blood cells of the test fish. On the 60th and 63rd day of observation, total erythrocytes, hematocrit levels, and hemoglobin began to increase in all treatments except the positive control. This indicates that gourami were able to recover after the *A. hydrophila* infection occurred. Research by Hamka *et al.* (2020) the application of probiotics *Bacillus megaterium* PTB 1.4 and *Pediococcus pentosaceus* E2211 in catfish feed can stimulate an increase in total erythrocytes, hematocrit, and hemoglobin in the blood after *A. hydrophila* infection.

In the research of Djauhari *et al.* (2016) also stated that the addition of probiotic bacteria *Bacillus* sp. NP5 to goldfish can increase total erythrocytes, hematocrit levels, and hemoglobin simultaneously, after *A. hydrophila* infection. Total leukocytes, phagocytic activity, and respiratory burst activity increased after 50 days of probiotic application and continued to increase until day 54 post-infection. Whereas in the positive control treatment until day 63, the values tended to be significantly higher ($p < 0.05$) than the other treatments. This can occur because white blood cells continue to phagocytose foreign bodies or pathogens that infect the host. And there is no additional immune system that can reduce the infection caused by *A. hydrophila*. In contrast to the single and multispecies probiotic treatments, gourami fed with supplemented probiotics have additional immunity, so that probiotic treatment can reduce infection from the *A. hydrophila* infection.

Total leukocytes are one of the important indicators of the non-specific immune system during inflammation, their number can be used as an indicator of the health status of fish. The increase in total leukocytes post-infection is related to the inflammatory response, leukocytes will be produced in greater numbers to limit infection from *A. hydrophila* bacteria. Leukocytes are blood cells that are responsible for phagocytosis. Phagocytosis is the body's initial defense process and plays a role in killing pathogenic microorganisms and regenerating damaged or dead tissue.

Phagocytes are cells that can perform phagocytosis, phagocytic cells are found in monocytes, neutrophils, and macrophages Awasthi *et al.* (2013) phagocytic activity data is presented in Figure 2f. Phagocytic cells will kill bacteria by producing reactive oxygen during the respiration process Uribe *et al.* (2011). According to Sugiani *et al.* (2013) that the higher the respiratory burst activity, the greater the production of free radicals used to fight pathogens. Total leukocytes, phagocytosis activity, and respiratory burst activity on day 57 in all probiotic treatments decreased compared to the positive control. This condition indicates that the fish are recovering from *A. hydrophila* infection.

This is supported by the opinion of Bunnoy *et al.* (2019), who used *Acinetobacter* KU011TH in catfish and Silarudee *et al.* (2019) stated that using *L. plantarum* CR1T5 in catfish can induce respiratory burst activity compared to the control.

The fish body's defense system against disease consists of a non-specific immune system and a specific immune system. The non-specific immune system is divided into a primary defense system or physical defense in the form of skin, scales, and mucus. Secondary defense or humoral mechanisms that are plasmatic, such as lysozyme, interferon, stomach acid, lactoferrin, and complement Sugiani *et al.* (2013).

Lysozyme is an important defense molecule in the form of a protein involved in the nonspecific immune system, including in fish and has lytic activity against Gram-positive and Gram-negative bacteria. Lysozyme can activate complement and phagocytic activity, and can break β glucoside and N-acetylmuramic bonds in peptidoglycan, so as to damage the bacterial cell wall (found in Gram-positive bacteria) Awasthi *et al.* (2013). Based on the results of measuring the lysozyme activity of gourami, all treatments showed an increase in value on day 54, this happened because *Bacillus* NP5 Rif^R and *L. plantarum* Cip^R are Gram-positive bacteria that can activate lysozyme by producing bactericides and plantaricin in *L. plantarum*, as natural antimicrobial agents. Nayak (2010) reported that single or multi-species application of probiotics can modulate the increase of lysozyme activity in teleost fish.

This can be seen from the high number of *A. hydrophila* Rif^R in the liver and kidney organs, therefore most of the enzymes produced in lysozyme activity are used to lyses pathogenic bacteria, which then lysozyme activity gradually begins to decrease on day 60 and day 63. This is supported by the opinion of Nasrullah *et al.* (2019) lysozyme activity in the kidney and liver organs of catfish after *A. hydrophila* infection increased and reached an optimum at the 12th hour, then gradually decreased to normal conditions. The application of single and multi-species probiotics can indirectly increase the resistance of gourami (Figure 3). This is indicated by the low abundance of *A. hydrophila* Rif^R bacterial cells in the liver and kidney organs which are lower ($p < 0.05$) compared to other treatments.

This is in line with the results of research by Thy *et al.* (2017), the application of probiotics *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B in catfish infected with *Edwardsiella ictaluri* can reduce pathogenic bacteria from the target organs. Probiotics are an alternative to inhibit pathogen infection by enhancing the host immune response by stimulating non-specific immunity (Fyzul *et al.*, 2014). In the opinion of

Simon *et al.* (2021) this condition occurs because probiotics and probiotic-generated components interact with gut associated lymphoid tissue (GALT) to induce host immune responses. Based on the histopathology results of gourami liver and kidney tissues, there was some damage that occurred in the K (+) treatment and probiotic application after *A. hydrophila* infection.

Gourami's kidney tissue in the K (+) treatment experienced severe necrosis of the renal tubules and widening of the tubule lumen causing hemorrhage. Inflammatory reactions appear due to infection caused by pathogenic bacteria *A. hydrophila*. This is in line with the research of Moustafa *et al.* (2020) that the kidney tissue of tilapia infected with *A. hydrophila* has inflammatory cell infiltration, necrosis in the renal tubules, accumulation of hyaline droplets in the tubular epithelium. Gourami liver tissue in the K (+) treatment experienced severe necrosis in the sinusoids, widening of the sinusoids caused congestion. Then granulomas and vacuolization form in liver tissue due to infection with pathogenic bacteria *A. hydrophila*.

This is in line with the opinion of Abdelhamed *et al.* (2017) catfish infected with *A. hydrophila* bacteria, congestion will appear in the sinusoids of the liver tissue then an inflammatory reaction occurs that modulates macrophages and lymphocyte cells to gather and form round structures called granulomas. Vacuolization is also seen as the presence of vacuoles in the cytoplasm of liver cells that enlarge and fill most of the cell volume due to infection with pathogenic bacteria *A. hydrophila*. The administration of probiotics was able to reduce lysis and damage to epithelial cells in the renal tubules, compared to K(+). The high level of damage to the liver and kidney tissues of gourami in the K(+) treatment caused the number of post-infection fish deaths to be higher than the other treatments. This is in accordance with the opinion of Laith and Musa (2013) that liver tissue infected with *A. hydrophila* shows several cells with necrosis vacuole damage (vacuolization).

The results of research by Afifi *et al.* (2000) explained that toxins produced by *A. hydrophila* and extracellular products such as hemolysin, protease, elastase can cause severe necrosis in the liver. Based on the results of immune response parameters including total erythrocytes, hematocrit levels, hemoglobin, total leukocytes, phagocytic activity, respiratory burst activity, lysozyme activity, and abundance

of *A. hydrophila* bacterial cells in target organs, as well as histopathology in target organs (liver and kidney) pre-infection and post-infection, probiotic application treatment is better than control treatment.

CONCLUSION

The application of probiotics through feed can work synergistically in improving growth performance, increasing gut microbiota abundance, immune response, and gourami resistance to *A. hydrophila* with the best results in the multispecies probiotic treatment (BL).

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