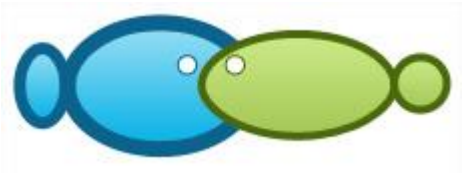

Isolation and identification of potential lactic acid bacteria as probiotics from the intestines of repang fish (*Puntioplites waandersi*)

Agustina Agustina, Gina Saptiani, Esti H. Hardi

Department of Aquaculture, Faculty of Fisheries and Marine Science, Mulawarman University, Samarinda, Indonesia. Corresponding author: A. Agustina, agustina@fpik.unmul.ac.id

Abstract. The present study aimed to isolate and characterize lactic acid bacteria (LAB) from the intestines of repang fish (*Puntioplites waandersi*), which have a potential as probiotic bacteria in vitro. The sample consisted of ten *P. waandersi* with a length of about 15.39±0.65 cm and a weight of about 40.04±6.24 g, taken from fishermen in the Mahakam River, Kutai Kartanegara Regency, East Kalimantan Province, Indonesia. The tests carried out included the isolation of LAB, characterization of isolates, antibacterial activity against pathogenic bacteria (*Aeromonas hydrophila*, *Pseudomonas* sp. and *Edwardsiella ictaluri)*, antibiotic sensitivity, tolerance to several media and enzymatic activity. Five isolates of LAB have been isolated: *Enterococcus* sp. (R1 and R2), *Lactobacillus* sp. (R3), and *Lactococcus* sp. (R4 and R5). The five LAB had strong antibacterial activity against pathogens, some were resistant to antibiotics and had a tolerance to bile salts, salinity and temperature, and exhibited amylolytic, proteolytic, and lipolytic activities, so these LAB have potential as probiotics.
Key Words: *Aeromonas hydrophila,* antibacterial activity, enzymatic activity, freshwater fish.

Introduction. Disease caused by infection with pathogenic bacteria is a problem that often causes losses in fish farming activities around the Mahakam River, Kutai Kartanegara Regency, East Kalimantan Province, Indonesia. The dominant bacteria found in sick and healthy fish were *Aeromonas hydrophila* and *Pseudomonas* sp. (Hardi & Pebrianto 2012; Agustina et al 2014). Mortality in fish infected by these two bacteria is quite high, reaching 50-80% (Hardi et al 2014; Agustina et al 2019). Other pathogenic bacteria that often cause disease in cultured freshwater fish such as catfish and tilapia (*Oreochromis niloticus)* are the *Edwardsiella ictaluri* bacteria. *E. ictaluri* was found to infect catfish such *Clarias* sp., with specific symptoms in the form of ulcers on the head, causing a mortality reaching 50% (Keskin et al 2004). Seasonal changes that occur affect water quality in the Mahakam River, along with increasing cases of bacterial diseases. Changes in water quality have an impact on decreasing the immune response of fish and, on the other hand, triggering the development of bacteria in the aquatic environment (Verma & Gupta 2015; Sunitha & Krishna 2016).

Efforts to control bacterial diseases in fish have been carried out for a long time. The utilization of probiotics is a strategy of disease control in aquaculture activities, especially those related to bacterial infections (Merrifield & Ringo 2014; Lee et al 2015; Caipang et al 2020; Hasan & Banerjee 2020). Lactic acid bacteria originating from fish intestines is a type of probiotic bacteria that has been proven to improve the fish health through a better use of nutrients and due to their antibacterial properties, increasing their immune response in dealing with pathogenic infections. Several studies have shown that groups of lactic acid bacteria (LAB) exhibit antibacterial activity in vitro, on rainbow trout (*Oncorhynchus mykiss*) (Balcázar et al 2008), tilapia (*O. niloticus*) (Zapata & Lara-Flores 2013), common carp (*Cyprinus carpio*) (Kaktcham et al 2017), several other freshwater fish species (Hanol et al 2020) and marine fish (Alonso et al 2019). The types of lactic acid bacteria that show potential as probiotics in fish include *Lactococcus lactis*, *Enterococcus* spp., *Lactobacillus plantarum* and *Leuconostoc mesenteroides* (Ringo et al

2018). The potential of local fish as a source of probiotic bacteria needs to be investigated, considering that this type of fish is still abundant in the waters of the Mahakam River and in the surrounding lakes. In kelabau fish (*Osteochilus melanopleurus*), several bacteria have a probiotic potential (Agustina et al 2018). Therefore, it is necessary to conduct a study on the isolation and characterization of LAB from the intestines of repang fish (*Puntioplites waandersi*). The in vitro antagonistic activity, tolerance to various media and enzymatic activity tests were carried out in this study as the initial stage of screening probiotic candidate bacteria in *P. waandersi*.

Material and Method

Isolation of lactic acid bacteria from fish intestines. *P. waandersi* was obtained from fisherman in Mahakam River, Kutai Kartanegara Regency, East Kalimantan Province. The present study used ten fish in health conditions with an average weight of 40.04±6.24 g and a length of 15.39±0.65 cm (Figure 1). Exploration research was selected for this study. Purposive sampling was carried out to get 10 *P. waandersi* individuals. Fish samples were carried in one plastic container with water and oxygen. Necropsy and isolation of LAB were conducted at the Laboratory of Aquatic Microbiology and Biotechnology, Faculty of Fisheries and Marine Sciences, Mulawarman University, Samarinda, East Kalimantan Province, Indonesia.

Figure 1. *Puntioplites waandersi* from Mahakam River (original photo).

Isolation of LAB from the fish intestine was conducted referring to Hanol et al (2020) and Patel et al (2020). The fish body surface was sterilized with 70% alcohol to avoid bacterial contamination. Then, 1 mL of homogenized intestinal contents was placed into a test tube containing 9 mL of sterile physiological solution, which was serialized and spread in de Man Rogosa and Sharpe Agar (MRSA) media, and finally the culture was incubated (Memert incubator) at a temperature of 37^{o}C for 24-48 hours. LAB was cultured on de Man Rogosa and Sharpe Broth (MRSB) media. The pathogens *A. hydrophila* and *Pseudomonas* sp. virulent strains were isolated from *O. niloticus* and a virulent strain of *E. ictaluri* was isolated from catfish (*Pangasius* sp.). Pathogens were cultured on Tryptic Soya Agar (TSA) media and Triptic Soya Broth (TSB) media.

Lactic acid bacteria characterization and identification. The LAB isolated from the intestines of *P. waandersi* were characterized based on their colony morphology and biochemical characteristics. Further identification was carried out referring to Bergey's Manual of Determinative Bacteriology (Holt et al 1994).

Antibiotic sensitivity test. The sensitivity of LAB isolates from the *P. waandersi* intestines to antibiotics was tested using the disc diffusion technique (Patel et al 2020). LAB isolates were inoculated into MRSA media with a concentration of 10^6 CFU mL^{-1}, allowed to dry for about 1 hour, and then placed an antibiotic disc on top. The antibiotics used consisted of six types, namely: Oxytetracycline (30 mcg), Nalidixic acid (30 mcg), Gentamycin (10 mcg), Ciprofloxacin (10 mcg), Chloramphenicol (30 mcg) and Norfloxacin (10 mcg). The culture was then incubated for 24 hours at 37^{o}C. The sensitivity of LAB to antibiotics was measured based on clear zones around the bacterial colonies.

Antagonistic activity test. The LAB isolated from *P. waandersi* intestines were tested in vitro for their potential in inhibiting the growth of three pathogenic bacteria, namely *A.*

hydrophila, *Pseudomonas* sp. and *E. ictaluri*. This test used the disc diffusion technique modification from Hanol et al (2020). The LAB isolates from *P. waandersi* have been cultured in MRSB media and three pathogenic bacteria have been cultured in TSB media: *A. hydrophila*, *Pseudomonas* sp. and *E. ictaluri,* with a concentration of 10^6 CFU mL^{-1}, which were spread on TSA media and allowed to dry for about 1 hour. LAB isolates with a concentration of 10^6 CFU mL^{-1} in 25 µm were impregnated on a sterile disk with a diameter of 6 mm and placed on TSA media previously cultured by each pathogenic bacterium. The culture was then incubated at 37°C for 24 hours. The ability to inhibit pathogenic bacteria was measured based on clear zones around bacterial colonies.

pH, bile salt, and NaCl tolerance test. The LAB isolates were tested for tolerance in media with pH 2, 4 and 8, according to Patel et al (2020). MRSB media with different pH, namely 2, 4 and 8, were prepared using 1% HCl and 1 N NaOH and put into a test tube and sterilized. 1% (v/v) LAB cultures were inoculated into MRSB media with different pH and incubated at 37°C for 24 hours. Bacterial growth was observed by assessing the turbidity (optical density) using a 600 nm spectrophotometer (Rey Leigh VIS 7220G). The tolerance test for bile salts used fresh cow bile (modified from Patel et al 2020) with concentrations of 1, 2 and 3%, while salt tolerance used NaCl with concentrations of 3, 5 and 7%. The sterile MRSB media was put in a test tube with bile salts and NaCl concentration according to the treatment. A total of 9.9 mL of MRSB media has been treated and then added 0.1 mL of LAB culture. The culture in the test tube was then incubated at 37°C for 24 hours. Bacterial growth was observed by assessing the turbidity (optical density) using a 600 nm spectrophotometer.

The ability to grow with different temperatures test. The cultures of 1 mL LAB were inoculated into 9 mL of MRSB broth and incubated for 24 hours at 37°C, then 1 mL of 9 mL of MRSB medium were inoculated and incubated again at 15, 25 and 45°C for 24 hours. Bacterial growth was observed by assessing the turbidity (optical density) using a 600 nm spectrophotometer according to Risna et al (2020).

Enzymatic activity test. In the assay of amylolytic activity, the LAB was inoculated on MRSA media containing 1% starch (Fossi et al 2005). LAB was inoculated into MRSB media and incubated for 24 hours at 37°C. The culture was then dripped onto a sterile paper disk and then placed on MRSA media, which contained 1% starch. The culture was then incubated for 24 hours at 37°C. The observation of the clear zone around the colony was carried out by adding Lugol to the media. The proteolytic activity test was carried out using 1% skim milk in MRSA media (Nespolo et al 2010). LAB was inoculated into MRSB media and incubated for 24 hours at 37°C. The culture was then dripped onto a sterile paper disk and placed on MRSA media which already contained 1% skim milk. The culture was then incubated for 24 hours at 37°C. A proteolytic activity was observed based on the clear zone around the paper disk. The lipolytic activity test was carried out by adding 2 mL olive oil in 100 mL of MRSA media. Liquid cultures of LAB that had previously been incubated in MRSB media were dripped onto sterile paper disks and then placed on a MRSA media containing olive oil. The cultures were then incubated for 24 hours at 37°C. The lipolytic activity of LAB was shown by a clear zone around the colony, indicating that the medium was soluble and hydrolyzed (Benson 2001). The hydrolysis index can be known based on the diameter of the clear zone formed in the media, with the formula: diameter of clear zone/diameter of bacterial colonies (Melliawati et al 2015).

Data analysis. All data obtained were analyzed descriptively in the form of tables and figures.

Results

Characterization and identification of lactic acid bacteria isolated from fish intestines. Isolation of LAB from the intestine of *P. waandersi* from Mahakam River, Kutai Kartanegara Regency, East Kalimantan Province resulted in five isolates (Table 1). Table 1 shows the morphological and biochemical characteristics of these bacteria. All

colonies of LAB that have been isolated are circular in shape, have a convex elevation and an entire edge with two colors, namely white and turbid white. Based on the proximity of biochemical characteristics, the LAB isolates in the study were closely related to *Enterococcus* sp. (R1 and R2), *Lactobacillus* sp. (R3) and *Lactococcus* sp. (R4 and R5).

Table 1

Characteristics of lactic acid bacteria from *Puntioplites waandersi* intestines

Characteristic	*R1*	*R2*	*R3*	*R4*	*R5*
Whole colony	Circular	Circular	Circular	Circular	Circular
Color	White	White	Turbid white	Turbid white	Turbid white
Elevation	Convex	Convex	Convex	Convex	Convex
Edge	Entire	Entire	Entire	Entire	Entire
Basic morphology	Coccus	Coccus	Bacillus	Coccus	Coccus
Arrangements	Streptococcus	Streptococcus	Diplobacillus	Diplococcus	Streptococcus
Gram stain	+	+	+	+	+
Motility	+	+	-	-	-
Catalase	-	-	-	-	-
Indole	-	-	-	-	-
Voges-Proskauer	-	+	+	+	+
Metil-red	-	-	+	+	+
Acid production from carbohydrate					
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Sucrose	+	+	+	+	+
Lactose	+	+	+	+	+
Sorbitol	+	-	+	+	+
Mannitol	-	-	+	-	+
H_2S production	-	-	-	-	-
Gas production	-	-	-	-	-
Genus	*Enterococcus*	*Enterococcus*	*Lactobacillus*	*Lactococcus*	*Lactococcus*

Antibiotic sensitivity test. The susceptibility of the LAB isolates from the intestines of *P. waandersi* to six types of commercial antibiotics can be seen in Table 2. Based on Mayer (2007), the sensitivity of bacterial isolates to antibiotics was categorized into three groups, depending on their inhibition zone: at 13 mm as resistant, at 14-18 mm as intermediate and at 19 mm as susceptible or sensitive. Five isolates of LAB were in these three categories, with a range of inhibition zones from 9.33 to 22.33 mm.

Table 2

Sensitivities of lactic acid bacteria from *Puntioplites waandersi* intestine to some commercial antibiotics

LAB isolates	*CIP*	*NOR*	*C*	*CN*	*NA*	*OT*
R1	R	I	I	S	R	R
R2	R	S	R	I	R	R
R3	S	S	S	S	R	R
R4	S	S	S	S	R	R
R5	S	I	S	I	R	R

CIP-Ciprofloxacin (10 mcg); NOR-Norfloxacin (10 mcg); C-Chloramphenicol (30 mcg); CN-Gentamycin (10 mcg); NA-Nalidixic Acid (30 mcg); OT-Oxytetracycline (30 mcg); R-Resistant; I-Intermediate, S-Sensitive.

Antagonistic activity test. Antagonistic tests of 5 LAB isolates were tested for their ability to inhibit the growth of 3 pathogenic bacteria, namely: *A. hydrophila, Pseudomonas* sp. and *E. ictaluri* (Table 3). The clear zone of all LAB isolates against the 3 pathogens ranged from 10 to 15.33 mm. An antibacterial activity that can produce an inhibition zone

between 10.0 to 20.0 mm is classified in the strong category (Davis & Stout 1971). The bacterial isolate R5 showed the largest size of the inhibition zone against *A. hydrophila*, which was of 15.33 mm, and the R4 isolate showed the smallest inhibition zone, of 11.00 mm. The largest inhibition zone against *Pseudomonas* sp., of 13.67 mm, was indicated by the isolate R2 and the isolate R1 showed the smallest inhibition zone, of 10.00 mm. Isolate R3 showed the largest inhibition zone against *E. ictaluri*, of 14.00 mm, and R2 of 11.00 mm, as the isolate with the smallest inhibition zone. The greatest inhibitory ability or antibacterial activity against the three pathogenic bacteria was shown by isolate R5 with the inhibition zone of 13.66 mm, followed by R3 of 12.56 mm and R2 of 12.33 mm.

Table 3

Diameter of the inhibition zone (mm) of lactic acid bacteria from *Puntioplites waandersi* intestines against pathogens

LAB Isolates	*A. hydrophila*	*Pseudomonas sp.*	*E. ectaluri*
R1	11.67	10.00	11.33
R2	12.33	13.67	11.00
R3	12.00	11.67	14.00
R4	11.00	12.67	13.00
R5	15.33	12.33	13.33

pH, bile salt, and NaCl tolerance test. The results showed that five isolates of LAB from the intestines of *P. waandersi* could still live in media with an acid-base pH (pH 2-8) (Table 4). The tolerance increased with increasing pH levels. Tolerance of LAB isolates from the intestines of *P. waandersi* fish was also shown to levels of 1-3% bile salts. The tolerance of five isolates of LAB from the intestines of *P. waandersi* to salt (NaCl) levels of 3-7% was indicated by the bacterial growth, although it decreased with the increase of the NaCl levels.

Table 4

Optical density (OD) values of lactic acid bacteria isolates from *Puntioplites waandersi* intestines in media with different pH, bile salt and NaCl levels

LAB isolates	*pH*			*Bile salt*			*NaCl*		
	pH 2	*pH 4*	*pH 8*	*1%*	*2%*	*3%*	*3%*	*5%*	*7%*
R1	0.08	0.76	0.93	0.82	0.92	0.93	1.41	1.38	0.90
R2	0.12	0.37	1.01	0.80	0.87	0.90	0.66	0.49	0.09
R3	0.11	0.28	0.96	0.78	0.89	0.93	1.47	1.37	0.52
R4	0.11	0.18	0.93	0.82	0.91	0.93	1.32	1.23	0.32
R5	0.09	0.20	0.90	0.71	0.73	0.82	1.48	1.34	0.53

The ability to grow with different temperatures test. Isolates of LAB isolated from the intestines of *P. waandersi* were able to live in the temperature range of 15-45^{o}C, although the colony decreased at 45^{o}C (Table 5). At the time of isolation of these bacteria from the *P. waandersi* intestines and of their incubation at 37^{o}C, the colony showed a good growth.

Table 5

Optical density (OD) value of lactic acid bacteria isolates from *Puntioplites waandersi* intestines in media with different temperature levels

LAB isolates	*15^{o}C*	*25^{o}C*	*45^{o}C*
R1	0.04	1.58	0.82
R2	0.07	0.70	0.48
R3	0.06	1.58	0.95
R4	0.03	1.45	0.96
R5	0.07	1.60	1.05

Enzymatic activity test. LAB isolated from the intestines of *P. waandersi* fish showed enzymatic activity in vitro test (Figure 2). These bacteria show that the clear zone size for amylolytic activity ranged between 1.22-1.62, the proteolytic activity ranged between 1.26-1.49, and the lipolytic activity ranged between 1.19-1.26. The enzymatic activity is important to know in the probiotic screening, as an illustration of the ability of microbes to improve the digestion of host nutrients.

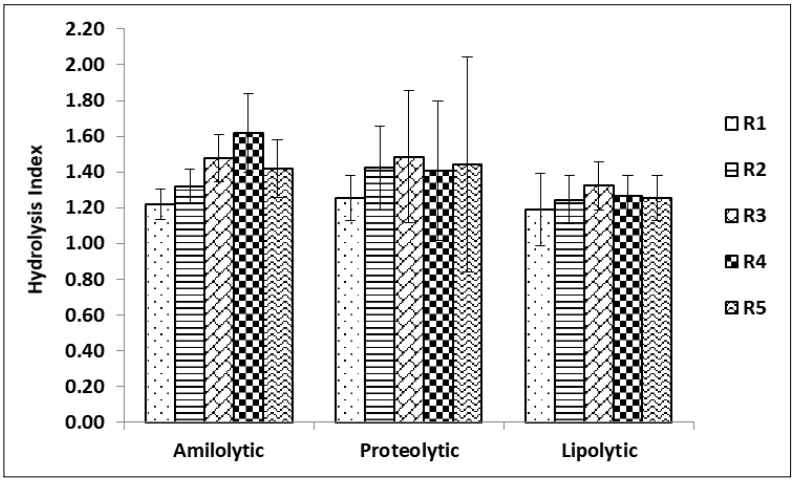

Figure 2. Enzymatic activity of lactic acid bacteria from *Puntioplites waandersi* intestines.

Discussion. These three types of LAB are commonly found in the digestive tract ofboth seawater fish and freshwater fish. Feliatra et al (2004) found that several bacteria isolated from the digestion of tiger grouper (*Epinephelus fuscoguttatus*) contained LAB from the genus *Lactobacillus*, *Bifidobacterium* and *Lactococcus*. In the digestive tract of star pomfret (*Trachinotus blochii*), the following isolates were found: B7-P4-K2 and B8-P4-K1, belonging to the genus *Lactobacillus,* and isolates B8-P4-K3 and 10-P4-K1, belonging to the genus *Enterococcus* (Irwansyah et al 2018).

Kumar et al (2013) also isolated LAB of the genus *Lactobacillus* sp. in five freshwater fish. The results were LAB of the genus *Lactobacillus* in all fish samples. Vijayaram et al (2016) also found several isolates of *Lactobacillus* spp. in the freshwater fish digestive tract. In addition, Wulandari et al (2015) found types of LAB, namely *Bacillus*, *Lactobacillus* and *Eubacterium,* in the digestive tract of the catfish. Species of *Lactococcus* have been found in the digestive tract of tilapia (*O. niloticus*) and can ferment industrial and agricultural wastes (Patel et al 2020).

All isolates of LAB from the intestines of *P. waandersi* were resistant to the antibiotics Oxytetracycline and Nalidix acid, while the antibiotics Norfloxin, Chloramphenicol and Gentamycin were classified as intermediate to sensitive. Resistance to antibiotics of LAB depends on the type and source of the isolate (Salminen et al 1998). The resistance of probiotic bacteria isolates is very helpful in concurrent application with antibiotics in the culture media. These bacteria will last a long time in the digestive tract of fish and are not affected by therapy with antibiotics. However, several isolates of LAB from fish intestines were also found that were sensitive to several types of antibiotics (Hanol et al 2020). *P. waandersi* used in this study were wild fish with a low chance of exposure to chemicals or antibiotics, so it was natural to find isolates from their intestines that were sensitive to antibiotics. This is in line with Agustina et al (2018) research on *O. melanopleurus*. Hanol et al (2020) found 25 isolates of LAB in several freshwater fish that could inhibit pathogenic bacteria with a range of weak to very strong inhibition zones. The best isolates were further identified as bacteria from the genera *Lactobacillus* and *Lactococcus*. This is in line with this research conducted on *P. waandersi*, genus *Lactococcus*, *Enterococcus* and *Lactobacillus,* which showed antibacterial activity in the strong range.

49 LAB isolates from the intestines of guppies (*Poecilia reticulata*) also showed strong antibacterial activity after being tested with the bacterium *Pseudomonas*

aeruginosa (ATCC 27853) in vitro (Jahangiri et al 2018). In vitro tests on LAB isolates from the intestines and gills of tilapia were in line with this study, suggesting the antibacterial ability of the LAB *Enterococcus faecalis* against several pathogens in fish (Prachom et al 2020). This is related to the ability of LAB to live and thrive in the digestive tract of fish, following the application of probiotics, given the acidic conditions in the fish's stomach. An important criterion for selecting LAB as probiotics is their potential viability at low pH (Kim & Austin 2008). This is in line with Vijayaram et al (2016) finding that bacterial isolates from several types of freshwater fish could live at a pH of 2-4.5 for 24 hours of incubation. On the contrary, Allameh et al (2012) found that isolates of LAB from the intestines of snakehead fish cannot live at pH 2 but at pH 3-8 with an incubation period of 2 hours. This was also reported by Jahangiri et al (2018), who found that incubation for 2 hours did not show any growth of LAB at pH 2.5. This is in accordance with Patel et al (2020) showing that isolates of LAB (*Lactococcus garviae*) can live in the range of 3-7% salt content, only decreasing at 7% salt content. NaCl is a substance that can inhibit the growth of several types of bacteria. In the present study, isolates of LAB were still able to live at a salt level of 7%, indicating that these bacteria are ideal as probiotic candidates. These results are in line with those found by Prachom et al (2020), who tested isolates of LAB in fresh bile. Within a concentration interval of 0-10%, the bacteria still grew well up to a bile level of 8% and then it decreased. Several studies reported that probiotics must survive or be resistant to substances that inhibit their growth in the digestive tract, such as bile salts (Allameh et al 2012; Prabhurajeshwar & Chandrakanth 2017). This is in line with Risna et al (2020), who found that the isolates of LAB from duck intestines could live at a temperature of 15-45^{o}C, optimally at 37^{o}C. It is suspected that LAB is mesophilic bacteria. However, there are indeed several species of this bacterium that can grow at a temperature of 45°C (Mulaw et al 2019). The presence of clear zones on amylolytic, proteolytic and lipolytic tests indicated that LAB isolated from the intestines of *P. waandersi* could lyse or degrade carbohydrates from flour, protein from skim milk and fat from olive oil. The presence of extracellular enzymes such as amylase, protease and lipase is a criterion that probiotics must possess to increase food digestibility. The enzymes produced by probiotics play an important role in the degradation of various food ingredients that are difficult for fish to digest. Balcázar et al (2006) found that the ability of probiotics to produce extracellular enzymes increase the food digestibility in the host. Isolates of LAB from the intestines of *P. waandersi* have a higher amylolytic and proteolytic hydrolysis index than their lipolytic hydrolysis index. This is associated with the tendency of *P. waandersi* in nature to eat a variety of foods (being omnivorous), such as plants and zoobenthos as the main food, and insects as complementary foods or plankton and worms as additional food (Kaban et al 2016). The digestive tract of fish contains microbial populations from the aquatic environment (Ganguly & Prasad 2012). The amylolytic and proteolytic hydrolysis index of the LAB from the intestines of *P. waandersi* was higher than in the study of Mulyasari et al (2016). They tested the enzymatic activity of bacteria from the intestines of gourami (*Osphronemus goramy*).

Conclusions. From the present study, it can be concluded that five isolates of LAB from the intestines of *P. waandersi* were biochemically identified as *Enterococcus* sp. (R1 and R2), *Lactobacillus* sp. (R3) and *Lactococcus* sp. (R4 and R5). Isolates of LAB from the intestines of *P. waandersi* showed a strong ability to control pathogenic bacteria *A. hydrophila*, *Pseudomonas* sp. and *E. ictaluri*. Also, they can tolerate wide ranges of pH, bile salts, NaCl and temperature values. Due to these characteristics and to their enzymatic activity in vitro, the isolates of LAB from the intestines of *P. waandersi* have a good potential as probiotics.

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Conflict of interest. The authors declare no conflict of interest.

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Authors:
Agustina Agustina, Department of Aquaculture, Faculty of Fisheries and Marine Science, Mulawarman University Gunung Kelua Campus, Jl. Gunung Tabur, Samarinda 75123, Indonesia, e-mail: agustina@fpik.unmul.ac.id
Gina Saptiani, Department of Aquaculture, Faculty of Fisheries and Marine Science, Mulawarman University Gunung Kelua Campus, Jl. Gunung Tabur, Samarinda 75123, Indonesia, e-mail: gina_saptiani@yahoo.com
Esti Handayani Hardi, Department of Aquaculture, Faculty of Fisheries and Marine Science, Mulawarman University Gunung Kelua Campus, Jl. Gunung Tabur, Samarinda 75123, Indonesia, e-mail: estieriyadi2011@gmail.com

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