Antagonistic activity of Kelabau fish (Osteochilus melanopleurus) gut bacteria against Aeromonas hydrophila and Pseudomonas sp.

by Agustina Agustina

Antagonistic activity of Kelabau fish (*Osteochilus melanopleurus*) gut bacteria against *Aeromonas hydrophila* and *Pseudomonas* sp.

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Abstract. The present study was conducted to isolate the kelabau fish (*Osteochilus melanopleurus*) gut bacteria, in order to identify and evaluate their potential for probiotics in freshwater aquaculture. A total of 19 bacterial isolates associated with *O. melanopleurus* gut, collected from Melintang Lake, East Kalimantan Province, Indonesia. Antibacterial activities were measured by the zone of inhibition in an antagonistic test against *Aeromonas hydrophila* (AH-1), *A. hydrophila* ATCC® 35654™* and *Pseudomonas* sp. (PS-1). Most of the extracelullar product (ECP) and intracelular product (ICP) of *O. melanopleurus* gut bacterial isolates demonstrated ability to prevent the growth of fish pathogens. The isolates that demonstrated high antagonistic activity were represented by isolates of BP1, BP2, BP3, BP4, BPs1, BPs2, BeP1 and BK. According to the biochemical characteristics of the eight isolates of *O. melanopleurus* gut from Melintang Lake East Kalimantan, revealed to be closely similar to *Aeromonas* sp., *Plesiomonas* sp., *Bacillus cereus*, *Acinetobacter* sp., *Pseudomonas pseudomallei*, *Vibrio alginolyticus*, *Bacillus circulans* and *Vibrio* sp. From this study, it can be concluded that the eight bacteria isolated from *O. melanopleurus* intestine, can be considered as potential candidates for probiotics development.

Key Words: digestive tract, probiotic, fish pathogens, antibacterial activity, freshwater aquaculture.

Introduction. Kelabau fish (*Osteochilus melanopleurus*) is one of the freshwater fish that widely spreads in Southeast Asia such as Malaysia, Burma and Indochina. In Indonesia *O. melanopleurus* are found in the islands of Sumatra and Kalimantan. This fish belongs to the Family Cyprinidae genus *Osteochilus* (Kottelat et al 1993). In East Kalimantan this fish is found in the middle of Mahakam River which is situated in Kutai Kartanegara Regency and West Kutai Regency. In addition, *O. melanopleurus* is also often found in several overflow lakes namely Lake Jempang, Semayang and Melintang.

O. melanopleurus is a herbivorous fish species, based on researches conducted by Aizam et al (1983) and Nasution & Nuraini (2004), who showed many plants, fruits, phytoplankton and algae in their intestine. The presence of microflora, especially bacteria in the digestive tract, was very instrumental in supporting fish health both in terms of nutrient utilization and enhancement of the immune system (Fuller 1992; Gatesoupe 1999; Nayak 2010; Merrifield & Ringo 2014). The ability of bacteria from the digestive tract of fish in suppressing the growth of pathogenic bacteria *Aeromonas hydrophila* has been observed in several other species such as common carp *Cyprinus carpio* (Chi et al 2014), *C. carpio* fry (Gupta et al 2014), *Cirrhinus mrigala* (Bhatnagar & Lamba 2015), and *Labeo rohita* (Ramesh et al 2015).

On the other hand, the use of cellular products from bacteria showed antagonistic ability against other bacteria. The study conducted by Hardi et al (2016) found that extracellular (ECP) and intracellular (ECP) products from *Pseudomonas* sp. were able to inhibit the growth of *A. hydrophila* bacteria in vitro. In the *C. carpio* intestine three strains of *Aeromonas veronii* BA-1, *Vibrio lentus* BA-2 and *Flavobacterium sasangense* BA-3 were found and their extracellular products were able to increase disease resistance (Chi et al 2014).

Studies about the composition of microflora in the intestine of *O. melanopleurus* and its ability to control pathogenic bacterial infections have never been published yet. On the other hand, the increase cultivation of several types of freshwater fish in Kutai Kartanegara Regency, East Kalimantan Province has the potential to experience Aeromonas septicemia or red spots caused by bacteria *A. hydrophila* and infection by *Pseudomonas* sp. (Hardi 2012; Hardi & Pebrianto 2012; Agustina et al 2014). *O. melanopleurus* has never been reported to been infected by both types of bacteria. This is the reason why the role of microbes in the digestive tract of *O. melanopleurus* and their potential as candidates of probiotics was interesting to be carried out.

Material and Method

Samples of Osteochilus melanopleurus and isolation of gut bacteria. *O. melanopleurus* was obtained from fisherman in Melintang Lake, Kutai Kartanegara Regency, East Kalimantan Province (Figure 1). Twenty fish in health condition with average weight of 56.51±26.08 g and a length of 17.20±2.43 cm were used in the present study (Figure 2). Exploration research was selected on this study. Purposive sampling was carried out to get 20 *O. melanopleurus* individuals. Fish samples were carried in two plastic containers containing water and oxygen. Necropsy and isolation of gut microflora was conducted at the Aquatic Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Mulawarman University, Samarinda, East Kalimantan Province, Indonesia.

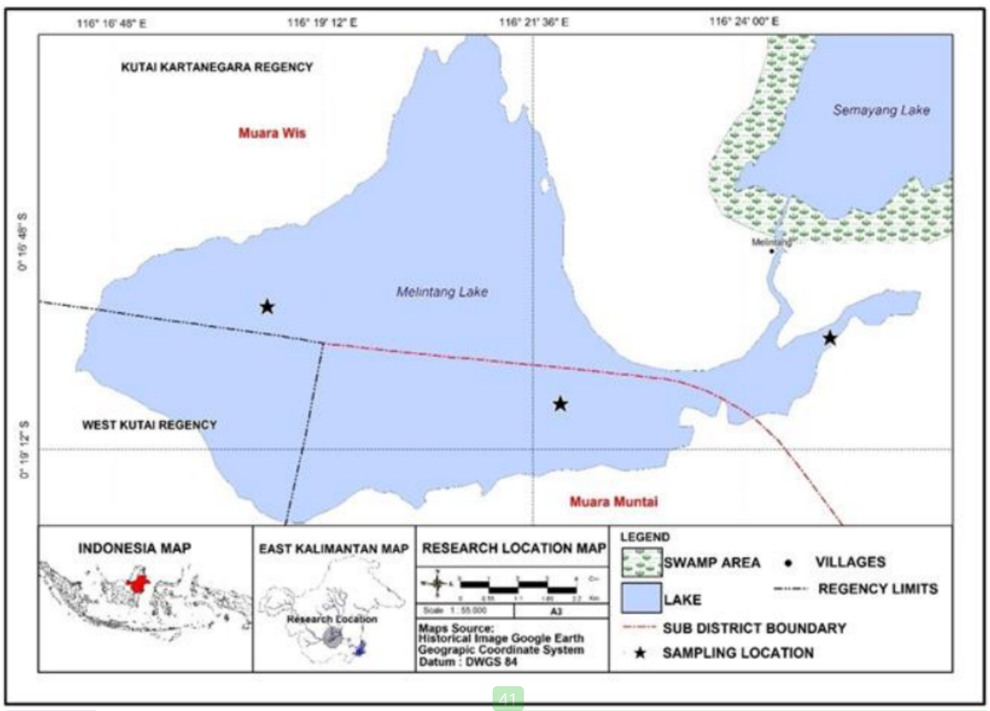

Figure 1. Sampling location, Melintang Lake Kutai Kartanegara Regency, East Kalimantan Province, Indonesia.

Figure 2. *Osteochilus melanopleurus* from Melintang Lake (original).

Isolation of bacteria from fish intestine was conducted according to modified Ramesh et al (2015). Fish body surface was sterilized with 70% ethanol to avoid bacterial contamination. *O. melanopleurus* intestine was removed from the abdominal cavity under sterile conditions, then the intestinal contents were taken out and homogenized. After that 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 Triptic Soya Agar media and that the culture were incubated in temperature of 30^{o}C for 24-48 hours.

The pathogen bacteria *Aeromonas hydrophila* (ATCC^R 35654 ^{TM*}) was purchased from ATCC, while *A. hydrophila* (AH-1), and *Pseudomonas* sp. (PS-1) virulent strains were isolated from *O. melanopleurus* Melintang Lake, Kutai Kartanegara Regency, East Kalimantan Province. Both were cultured on Triptic Soya Broth (TSB) and TSA media.

Antibiotic sensitivity test. Sensitivity of nineteen bacterial isolates from *O. melanopleurus* intestine were tested for their susceptibility to six commercial antibiotics namely Ciprofloxacin (10 mcg), Nalidixic Acid (30 mcg), Oxytetracycline (30 mcg), Norfloxacin (10 mcg), Nitrofurantoin (100 mcg), and Gentamicin (10 mcg). Subsequently, 100 µL of each of nineteen bacterial isolates from *O. melanopleurus* intestine (10^6 CFU mL^{-1}) was distributed to TSA media. An antibiotic disk was placed on the agar surface and incubated for 24 hours at 30^{o}C. After incubation, the clear area of the inhibition zone was measured according to Ramesh et al (2015).

Cellular component preparation. Two antigenic components from nineteen *O. melanopleurus* intestinal bacterial isolates, namely intra cellular product (ICP) and extracellular product (ECP) were prepared by the method of Das et al (2006).

Antagonistic activity test. Nineteen bacteria isolated from *O. melanopleurus* intestine and their internal and external cellular components were tested for their potential in inhibiting the growth of three pathogenic bacteria namely *A. hydrophila* (AH-1), *A. hydrophila* ATCC^R 35654 ^{TM*} and *Pseudomonas* sp. (PS-1) in vitro. This test used the Kirby-Bauer method (disc diffusion technique). Nineteen bacterial isolates from *O. melanopleurus* intestine and three pathogenic bacteria have been cultured in TSB media. *A. hydrophila* (AH-1), *A. hydrophila* ATCC^R 35654 ^{TM*} and *Pseudomonas* sp. (PS-1) with a concentration of 10^6 CFU mL^{-1} were spread on TSA media. Intestinal bacterial isolates with a concentration of 10^6 CFU mL^{-1} in quantity of 25 µm were impregnated on a sterile disk with a diameter of 6 mm and placed on TSA media previously cultured by each pathogenic bacteria. The culture was then incubated at 30^{o}C for 24 hours. The ability to inhibit pathogenic bacteria was measured based on clear zones around bacterial colonies and reported in mm (Hardi et al 2016). The same method was applied to the ICP and ECP inhibitory tests of bacteria from the intestine of *O. melanopleurus*.

Interaction test between eight bacterial isolates. Eight bacterial isolates which showed the best results in the previous test, namely BP1, BP2, BP3, BP4, BeP1, BPs1, BPs2 and BK were tested for their each other interaction. This test has used the Kirby-Bauer method (disc diffusion technique). Eight bacterial isolates that have been cultured in TSB media were taken 10^6 CFU mL^{-1} each then spread on TSA media. The other bacterial isolates with a concentration of 10^6 CFU mL^{-1} as much as 25 µm were impregnated on a sterile disk with a diameter of 6 mm and placed on TSA media previously cultured by each bacterial isolates. The culture was then incubated at 30°C for 24 hours. Interaction between the eight bacterial isolates was measured based on clear zones around bacterial colonies and the diameter was expressed in mm. Antagonistic interaction was observed in the clear zone around the bacterial colonies.

Identification. Eight bacterial isolates from *O. melanopleurus* intestine selected based on the ability to inhibit the three pathogenic bacteria were characterized based on biochemistry including gram staining, catalase, motility, oxidase, carbohydrate fermentation based on Bergey's Manual Systemic Bacteriology (Holt et al 1994). The hydrolysis ability of casein and starch was analyzed by agar diffusion method.

Results and Discussion

Isolation of gut bacteria. Isolation of bacteria from the intestine of Kelabau fish from Melintang Lake, Kutai Kartanegara Regency, East Kalimantan Province resulted in 19 isolates (Table 1). Table 1 shows the morphological characteristics of bacterial colonies; there are three types of colors, namely yellow, white and turbid white with flat and convex characters. The shape of the colony consists of circular, irregular, filamentous and rhizoid.

Table 1

Morphological features of *Osteochilus melanopleurus* gut bacterial colonies from Melintang Lake, Kutai Kartanegara Regency, East Kalimantan Province

No	*Color*	*Form*	*Character*	*Isolates*
1	Yellow	Iregular	Flat	MK
2	White	Circular	Convex	BP1
3	White	Circular	Flat	BP2
4	White	Circular	Convex	BP3
5	White	Circular	Flat	BP4
6	Turbid white	Circular	Flat	BPs1
7	Turbid white	Circular	Convex	BPs2
8	Turbid white	Circular	Flat	BPs3
9	Turbid white	Circular	Flat	BPs4
10	Turbid white	Circular	Convex	BPs5
11	White	Rhizoid	Flat	MaP
12	White	Circular	Convex	BeP1
13	White	Circular	Convex	BeP2
14	Yellow	Circular	Flat	BK
15	White	Filamentous	Flat	KP
16	White	Iregular	Convex	MP
17	Yellow	Circular	Convex	BeK
18	Turbid white	Iregular	Convex	MPs
19	Yellow	Rhizoid	Flat	MaK

Antibiotic sensitivity test. Susceptibility of bacterial isolates from the intestine of *O. melanopleurus* to six types of commercial antibiotics can be seen in Table 2. The commercial antibiotic used as a positive control in this study was Oxytetracycline. All commercial antibiotics tested showed a sensitive category to nineteen bacterial isolates from *O. melanopleurus* intestine with clear zones with a range of 13.00-38.50 mm. The sensitivities of bacterial isolates in this study have referred to the inhibition category of several antibiotics reported by Mayer (2007).

Table 2

Sensitivities of bacteria from intestine of *Osteochilus melanopleurus* to some commercial antibiotics

Isolates	*Inhibition zone (mm)*						*Characteristic activity*
	CIP	*NA*	*OT*	*NOR*	*F*	*CN*	
MK	33	30	26	26	20	25	Sensitive
BP 1	36	23	30	27	20	22	Sensitive
BP 2	30	31	23	25	22	20	Sensitive
BP 3	31	31	27	27	22	19	Sensitive
BP 4	29	28	27	13	22	20	Sensitive
BPs1	31	25	28	25	17	22	Sensitive
BPs2	35	22	31	31	17	18	Sensitive
BPs3	38	22	22	31	17	20	Sensitive
BPs4	36	28	13	31	18	23	Sensitive
BPs5	34	26	25	25	18	20	Sensitive
MaP	31	28	31	30	25	23	Sensitive
BeP1	36	24	29	29	17	22	Sensitive
BeP2	32	26	24	28	14	20	Sensitive
BK	34	29	29	26	16	15	Sensitive
KP	36	35	27	38	24	30	Sensitive
MP	35	29	31	30	24	21	Sensitive
Bek	33	17	16	30	20	19	Sensitive
MPs	28	22	19	27	17	22	Sensitive
Mak	35	33	30	32	19	24	Sensitive

CIP: Ciprofloxacin (10 mcg), Na: Nalidixic Acid (30 mcg), OT: Oxytetracycline (30 mcg), NOR: Norfloxacin (10 mcg), F: Nitrofurantoin (100 mcg), CN: Gentamicin (10 mcg).

These results showed that bacteria from *O. melanopleurus* intestine have a high sensitivity to commercial antibiotics that are widely used in controlling bacterial diseases in fish farming. The *O. melanopleurus* of this study were wild fish that had not been exposed to chemicals such as the commercial antibiotics tested. The sensitivity of bacterial isolates from wild fish intestines in high waters to several commercial antibiotics was also observed by Ramesh et al (2015) and Guo et al (2018).

Antagonistic test. Antagonistic test of nineteen bacterial isolates and two cellular components were tested for their antogonistic ability on three pathogenic bacteria, namely *A. hydrophila* (AH-1), *A. hydrophila* ATCC[R] 35654 [TM*] and *Pseudomonas* sp. (PS-1) which was able to inhibit the growth of pathogenic bacteria (Table 3).

In Table 3 can be seen that intestinal bacteria (IB) from *O. melanopleurus* intestine has antagonistic ability against *A. hydrophila* (AH-1) with clear zone ranging from 8.0 to 13.0 mm. IB antagonistic activity was higher compared to ICP and ECP antimicrobial activities, namely 6.0-11.0 mm and 7.0-9.0 mm respectively.

In general, cellular components of bacteria derived from *O. melanopleurus* intestines also showed antagonistic ability against *A. hydrophila* ATCC[R] 35654 [TM*]. BP3 demonstrated highest ability to inhibit *A. hydrophila* ATCC[R] 35654 [TM*] with a clear zone of 13 mm, while BPs4, MaP, BeP2, MP and MPs isolates did not show antagonistic ability against bacteria *A. hydrophila* ATCC[R] 35654 [TM*] (6.0 mm). However, all ECPs from Kelabau bacterial fish gut showed antagonistic ability against three pathogenic bacteria with clear zones ranging from 7.0 to 10.0 mm except MK. The same aspect was also shown by ICP with a clear zone ranging from 7.0 to 11.0 mm.

Table 3

Diameter inhabitation zone (mm) of intestinal bacteria (IB), intracellular product (ICP) and extracellular product (ECP) bacteria from intestine of *Osteochilus melanopleurus* against pathogens

Isolates	*A. hydrophila (AH-1)*			*A. hydrophila* ATCC® *35654* TM			*Pseudomonas sp. (PS-1)*		
	IB	*ICP*	*ECP*	*IB*	*ICP*	*ECP*	*IB*	*ICP*	*ECP*
MK	9.0	6.0	9.0	10.0	7.0	8.0	10.0	6.0	6.0
BP 1	13.0	8.0	8.0	11.0	8.0	8.0	10.0	9.0	8.0
BP 2	8.0	8.0	7.0	12.0	8.0	8.0	10.0	9.0	8.0
BP 3	11.0	9.0	8.0	13.0	8.0	10.0	9.0	10.0	9.0
BP 4	9.0	7.0	8.0	10.0	8.0	9.0	8.0	7.0	8.0
BPs1	10.0	6.0	8.0	10.0	8.0	9.0	11.0	6.0	8.0
BPs2	10.0	6.0	7.0	11.0	7.0	9.0	9.0	7.0	8.0
BPs3	9.0	6.0	8.0	10.0	7.0	8.0	9.0	7.0	8.0
BPs4	9.0	7.0	8.0	6.0	8.0	9.0	9.0	6.0	8.0
BPs5	9.0	7.0	8.0	9.0	7.0	8.0	8.0	7.0	9.0
MaP	12.0	7.0	7.0	6.0	8.0	9.0	7.0	7.0	8.0
BeP1	12.0	9.0	8.0	10.0	8.0	7.0	10.0	10.0	10.0
BeP2	12.0	7.0	8.0	6.0	7.0	8.0	8.0	7.0	7.0
BK	13.0	7.0	9.0	10.0	8.0	10.0	9.0	9.0	9.0
KP	10.0	7.0	8.0	8.0	7.0	8.0	8.0	7.0	8.0
MP	11.0	7.0	8.0	6.0	8.0	9.0	8.0	8.0	7.0
Bek	11.0	11.0	7.0	9.0	9.0	8.0	9.0	8.0	7.0
MPs	11.0	7.0	8.0	6.0	7.0	8.0	9.0	7.0	8.0
Mak	10.0	9.0	8.0	8.0	8.0	7.0	7.0	8.0	7.0

The results indicated that bacterial cells and cellular components of *O. melanopleurus* gut microflora have varying abilities in inhibit pathogens. Diameter inhabitation showed medium to strong inhabitation that ranged from 6.0 to 13.0 mm. An antibacterial activity which is able to produced inhabitation zone between 5.0 and 10.0 mm is classified as medium category and from 10.0 to 20.0 mm is classified as strong category (Davis & Stout 1971).

The antagonistic test conducted in this study proved that bacterial cells have the ability to inhibit pathogenic bacteria in vitro. These results are supported by several previous studies such as Agustina (2007), Agustina et al (2010), Bhatnagar & Lamba (2015), Prayitno et al (2015), Ramesh et al (2015), and Mourino et al (2016). The bacterial cell component has the potential to inhibit pathogenic bacteria as reported by Das et al (2006) and Hardi et al (2016).

Based on the antagonistic ability of nineteen isolates above, eight bacterial isolates which had the largest clear zone in inhibiting three pathogenic bacteria were selected for further study. These bacteria were BP1, BP2, BP3, BP4, BPs1, BPs2, BeP1 and BK.

Interaction test between eight bacterial isolates. The antagonistic interaction between eight selected bacterial isolates is shown in Table 4. Table 4 shown that antagonistic interaction between isolates were ranged from 7.00 to 12.00 mm. These results indicated that eight bacterial isolates from *O. melanopleurus* gut have medium to strong antagonistic activity (Davis & Stout 1971).

Antagonistic interactions of these bacterial isolates showed that they were able to produce antibacterial compounds that could inhibit the growth of other bacteria. This includes several factors that influence the process of bacterial attachment and colonization of the digestive tract of fish (Ringo et al 2007).

Table 4

Diameter inhabitation zone (mm) between eight bacterial isolates from intestine of *Osteochilus melanopleurus*

Isolates	*BP1*	*BP2*	*BP3*	*BP4*	*BeP1*	*BPs1*	*BPs2*	*BK*
BP1	-	8.0	8.0	8.0	10.0	9.0	8.0	10.0
BP2	9.0	-	9.0	10.0	9.0	10.0	7.0	9.0
BP3	8.0	8.0	-	9.0	8.0	8.0	7.0	9.0
BP4	8.0	8.0	8.0	-	8.0	7.0	8.0	7.0
BeP1	10.0	8.0	8.0	9.0	-	8.0	8.0	8.0
BPs1	9.0	8.0	8.0	8.0	8.0	-	8.0	7.0
BPs2	8.0	8.0	8.0	8.0	8.0	8.0	-	8.0
BK	10.0	9.0	8.0	7.0	12.0	10.0	9.0	-

Identification. Biochemical characterization of eight best bacteria that have antagonistic ability against pathogenic bacteria *A. hydrophila* (AH-1), *A. hydrophila* ATCC[R] 35654 [TM*] and *Pseudomonas* sp. (PS-1) is shown in Table 5.

Based on the proximity of biochemical characteristics, the eight selected bacterial isolates in this study were closely related to *Aeromonas* sp. (BP1), *Plesiomonas* sp. (BP2), *Bacillus cereus* (BP3), *Acinetobacter* sp. (BP4), *Pseudomonas pseudomallei* (BPs1), *Vibrio alginolyticus* (BPs2), *Bacillus circulans* (BeP1) and *Vibrio* sp. (BK).

Table 5

Biochemical characteristics of eight *Osteochilus melanopleurus* gut bacteria

Characteristic	*BP1*	*BP2*	*BP3*	*BP4*	*BPs1*	*BPs2*	*BeP1*	*BK*
Gram stain	-	-	+	-	-	-	+	-
Motility	+	+	+	-	+	-	+	+
Catalase	+	+	+	+	+	+	+	+
Indole	+	+	-	-	-	+	-	-
Voges-Proskauer	+	-	+	+	-	+	-	-
Citrate	+/-	-	+/-	+	+	+	-	-
Oxidase	+	+	-	-	+	+	-	+
Starch hydrolysis	-	+	+	-	-	-	+	+
Casein hydrolysis	+	-	+	-	-	-	+/-	-
Gelatin hydrolysis	+	-	+	-	+	+	+	+
Grows at 7% NaCl	-	-	+/-	-	-	+	-	+
Acid production from carbohydrate								
Lactose	+/-	+	-	-	-	-	+	+
Glucose	+	+	-	-	-	+	-	+
Sucrose	+	-	+	-	-	+	+	+
Enzyme activity								
Amylase	-	+	-	-	-	-	+	+
Protease	-	-	+	-	-	-	-	-
Haemolytic activity	+	-	+	-	-	-	+	-

The eight isolates bacteria that show the best ability to inhibit pathogenic bacteria in this study are commonly found in fish and aquatic environments (Holt et al 1994). Gram-negative bacteria were found to be the predominant species in this research. This was in line with reports of Ringo et al (1995), Ringo & Gatesoupe (1998) and Ringo & Birkbeck (1999).

Sugita et al (1996) further reported that the intestinal bacteria from freshwater cultured fish showed antagonistic ability to some pathogenic bacteria such as, *Aeromonas* and *Pseudomonas* species. In Tilapia (*Oreochromis niloticus*) for example, *Bacillus pumilus* bacteria has been isolated and has potential as probiotics in controlling *A. hydrophila* infections (Aly et al 2008). Wu et al (2015) also reported that intestine of grass carp (*Ctenopharyngodon idella*) contained several genera of bacteria such as

Shewanella xiamenensis A-1, *S. xiamenensis* A-2 and *Aeromonas veronii* A-7 that have potential as probiotics. Whilst in the intestines of rainbow trout (*Oncorhynchus mykiss*) was found several types of bacteria which are able to inhibit several bacteria from Family Aeromonadaceae, Enterobacteriaceae, Bacilaceae (Araujo et al 2015).

The eight bacterial isolates have a potential as probiotics however further series of tests were required to achieve best probiotic product that able play an important role in the development of freshwater aquaculture.

Conclusions. From the present study, can be concluded that:
(1) Nineteen bacterial isolates showed medium to strong antagonistic ability to control pathogenic bacterial infections against *A. hydrophila* (AH-1), *A. hydrophila* ATCC[R] 35654 [TM*] and *Pseudomonas* sp. (PS-1) in vitro.
(2) Similarly, intra cellular products (ICP) and extracellular products (ECP) from nineteen bacterial isolates were also able to inhibit these three bacterial fish pathogen in vitro.
(3) Eight bacterial isolates showed the greatest antagonistic ability against pathogenic bacteria, *Aeromonas* sp. (BP1), *Plesiomonas* sp. (BP2), *Bacillus cereus* (BP3), *Acinetobacter* sp. (BP4), *P. pseudomallei* (BPs1), *V. alginolyticus* (BPs2), *B. circulans* (BeP1) and *Vibrio* sp. (BK). These bacteria have a potential to be probiotics.

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