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Isolation, Selection and Application of Probiotic Bacteria for Improvement the Growth Performance of Humpback Groupers (*Cromileptes altivelis*)

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Abstract

This study aimed to isolate and select probiotic bacteria from the digestive tract of the humpback grouper (*Cromileptes altivelis*) and the effect the selected probiotic bacteria had on the humpback groupers' growth performance. Fifty eight bacteria were successfully isolated and were selected based on their ability to hydrolyze starch, milk, and fat. In the selection phase, 9 bacterial isolates were selected. Re-selection was conducted based on amylolytic, lipolytic, and proteolytic properties. Based on the results of the enzyme activity test, 6 isolates which had the highest enzyme activity, i.e. isolates RM2, RM3, RM4, RM5, RM7, and RM8, were selected. This was followed by other tests, the antagonicity test, pathogenicity test, acid-base resistance test, adhesion test, and bacterial growth phase test. This testing phase resulted in 4 bacterial isolates, i.e. RM3, RM4, RM5 and RM 7 bacteria. The four bacteria were used in *in vivo* tests in humpback grouper (4.65±0.44 g), administered through feed for 40 days. The results of this study showed that fish fed the probiotic bacteria RM3, RM4, and RM 5 had significantly increased growth rates, decreased Feed Conversion Rates (FCR), and increased protein and fat retention ($P<0.05$). However, the growth performance in the treatment using probiotic bacteria RM7 did not show a significant difference ($P>0.05$) from the control.

Keywords: Growth performance; Humpback groupers; Probiotic

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1. Introduction

The humpback grouper (*Cromileptes altivelis*) is an important species in the grouper family in the Asia-Pacific region, especially in Indonesia, because of the high export demand and price [1]. The cultivation of groupers is concentrated in Asia, with China, Taiwan and Indonesia collectively supplying more than 90% of the total global production [2]. However, the intensive humpback grouper production is impeded by the slow growth rate. According to [3], growth rate of humpback groupers from 10 g to 500 g requires 14 months. The fish slow growth could be because the fish nutritional needs are not fulfilled or because the fish are unable to utilize the energy and nutritional substances in the feed. High-quality feed can be made by using high-quality raw materials or by adding enzymes which could break down macro nutrients in the feed.

The application of probiotics is one strategy that could be employed in modulating the composition of the intestinal micro biota which could help improve the host growth and digestion. Probiotics are supplementary microbes which have a positive effect on the host by increasing feed nutritional value and improving the host immune response towards disease [4]. Several researches have proven that probiotic supplementation can reduce operational costs in cultivation through growth and feed-utilization efficiency improvement [5, 6, 7]. In addition, the use of probiotics could also reduce the use of hazardous antimicrobial substances and improve appetite in cultivated species in a more sustainable and eco-friendly way [8, 9].

The selection of the probiotic bacteria is a major factor in determining the success in the application of probiotic in aquaculture [10]. The probiotic must have the following criteria: able to colonize, able to establish and multiply in the host gut, and able to produce extracellular digestive enzymes [11]. Several kinds of commercial probiotics can already be applied in fish cultivation; however, these probiotics are relatively less effective because of the difficulty maintaining the the number of bacteria at the optimum concentration in the intestines [12]. Therefore, isolation of probiotic bacteria from a similar species could increase the success rate in the host [4]. The strategy of isolating endogenous probiotics from a host's intestines and using them in the same species has been successfully implemented in fish [13]. Endogenous probiotics are a natural part of the micro flora system found in the digestive tract of living organisms.

The use of probiotics in aquaculture is currently focused on the probiotics' ability to improve the immune response and nutritional parameters through efficiency and feed conversion ratios. Studies about the relationship between probiotics and digestive enzyme activity are very limited. Some researchers have conducted enzyme activity-based studies on probiotics, i.e. in the Indian white shrimp *Fenneropenaeus indicus* [14], *Penaeus vannamei*, [15], the gilthead sea bream *Sparus aurata* L. larvae [16] and the rainbow trout *Onchorhynchus mykiss* [17]. Even though the role of probiotics has been studied in several aquatic organisms, there have been no studies about the effect of probiotics on enzyme activity and growth performance of humpback grouper (*C. altivelis*) which is the most valuable species in the grouper family. Therefore, this study aimed to isolate and screen probiotic candidates from the humpback grouper's digestive tract and study their effects on digestive enzyme activity and fish growth.

2. Materials And Method

2.1. Bacteria Isolation

The groupers used in this study came from two different areas, the Seribu Isles and Lampung. The four samples from the Seribu Isles taken from raising cages were ≥ 100 g. The samples from Lampung were taken from a hatchery: 5 fry (± 10 g) and 4 raising size fish (≥ 100 g).

The contents of the humpback grouper's intestinal tract were used as a source of the inoculum. The digestive organs (the stomach and intestines) from fry and mature humpback grouper were taken. The intestines were ground and each 1 g intestine was diluted with 9 mL sterile physiological solution (NaCl 0.85%). Serial dilution was done from 10^{-2} to 10^{-4}. The inoculum was cultured using the spread plate method on Sea Water Complete media (SWC; Bacto peptone 0.05g, yeast extract 0.01g, glycerol 0.03 ml, sea water 75 ml, distilled water 25 ml, bacto agar 1.5g) which had 2% starch (w/v), 2% skim milk (w/v), and 2% olive oil (v/v) added respectively. The cultures were incubated at 29^{o}C for 24 hours. Single colonies growing on the culture media which had different profiles were cultured repeatedly to obtain pure single inoculates [18]. The microbial isolates which had amylolytic, proteolytic, and lipolytic activity were selected using the selective method referring to the method used in terrestrial animals [19] combined with procedures for microbe isolation from the digestive tract of fish [20, 21, 22, 23].

2.2. Selection of the probiotic bacteria candidates

Selection of the probiotic bacteria candidates was aimed to find bacteria that had potential as a probiotic. The selection was done through these selection phases: 1) amylolytic, proteolytic, and lipolytic activity; 2) extracellular amylase, protease, and lipase enzyme activity; 3) bacteria antagonistic and pathogenic activity; 4) resistance to stomach acids and bile salts; 5) adhesion; 6) bacteria growth phase; 7) and pathogenicity.

2.2.1 Proteolytic, lipolytic, and amylolytic activity testing

The purpose of this testing was to measure the proteolytic, lipolytic, and amylolytic activity of each isolate through their ability to hydrolyze carbohydrate, protein, and fat. The probiotic bacteria candidates were grown on SWC media which had each been mixed with 2% (v/w) starch for the amylolytic test, 2% (v/w) skim milk for the proteolytic test, and 2% (v/v) olive oil for the lipolytic test. The isolate's ability to hydrolyze protein is signified by a transparent zone around the isolate which had been grown on the agar medium in which skim milk had been added. Fat hydrolization is signified by a green hue on the media in which olive oil had been added after being steeped in saturated copper sulfate ($CuSO_4$). The ability to hydrolyze carbohydrate is signified by the formation of a yellow zone around the colony growing after the medium is steeped in the reagent Potassium iodide (KI) 1%.

2.2.2. Protease, lipase and amylase enzyme activity testing

The bacteria were prepared for the measurement of enzyme activity by inoculating the microbe to 10 mL of SWC medium, and then incubated in a waterbath shaker at 29^{o}C at 140 rpm for 24 hours. The inoculum was then centrifuged at 11,000 rpm for 20 minutes at 4^{o}C [24]. The crude enzyme extract filtrate was then taken for amylase enzyme activity testing using 1% starch as the substrate [25], protease enzyme activity testing using the Bergmeyer and Grassi [26] method with casein as the substrate, and lipase enzyme activity using olive oil emulsion as the substrate [27].

2.2.3. Antagonistic activity testing

The probiotic bacteria candidates antagonistic activity (inhibition) towards *Vibrio alginolyticus* was tested *in vitro* using the co-culture method. The *V. alginolyticus* bacteria were marked with rifampicin resistance markers, 50 µg/mL (Va^{rif}). The probiotic bacteria candidates and Va^{rif} bacteria were each cultured in SWC-broth media for 24 hours in a waterbath shaker at 29^{o}C, 140 rpm. One hundred µL of the probiotic candidate inoculum at a density of 10^6 CFU/mL and Va^{rif} 10^3 CFU/mL each were put into 10 mL of the SWC broth medium and incubated again for 24 hours in a waterbath shaker at 29^{o}C, 140 rpm. As a control, the Va^{rif} bacteria plus sterile physiological solution (NaCl 0.85%) were grown on similar media. The Va^{rif} bacteria which grew on the TCBS ^{rif} medium were counted using the plate count method [18].

2.2.4. Pathogenicity testing

Pathogenicity testing was done to discover whether the probiotic bacteria candidates were pathogenic. The probiotic candidate isolates were injected to humpback groupers (weighing an average of 4.65 ± 0.44 g) intramuscularly at a concentration of 10^6CFU/mL at a dose of 1 mL. As the positive control, humpback groupers were injected with 1 mL of the *V. alginolyticus* pathogen at a concentration of 10^6 CFU/mL. Negative controls were injected with 1 mL phosphate buffer solution. After being injected, the fish were kept in 60 x 30 x 30 cm aquariums at a density of 5 fish per aquarium. The observation of the survival rate was done for 10 days.

2.2.5. Resistance to stomach acids and bile salts testing

The microbial isolates resistance to stomach acids and bile salts was evaluated to gauge their ability to survive in the stomach which has a low pH and to survive bile salts found in the anterior section of the intestines. The testing was done using the method in [28]. This method was done by inoculating 1.0 mL of the microbial isolate in a series of test tubes containing 9 mL sterile medium solution at a pH of 2.5 (the pH was regulated by adding HCl) and a pH of 8.5 (the pH was regulated by adding NaOH), and incubating them at 29°C. Observations were made after 2, 4, 6, and 8 hours post inoculation and the number of microbes was calculated using the spread plate method [18].

2.2.6. Adhesion testing

The attachment or adhesion testing was done using the method stated in [29], i.e. using a stainless steel plate. The test was by placing the plate in 250 mL of growth medium which had been inoculated with 1 mL of the microbial culture in a 1 L Erlenmeyer, then incubated at 29°C for 24 hours. The density of the biofilm was

analyzed after 24 hours by washing the plate using phosphate buffer solution (BF). And then, the plate was swabbed thoroughly. The swab was placed in a test tube containing 10 mL BF and the tube was vortexed for 1 minute. After that, the number of bacteria was counted using the plate count method (CFU/cm^2). The number of microbes growing in the liquid phase was also calculated by taking 1 mL of the growth medium and diluting it with 9 mL BF solution. And then the microbes were cultured and the number of microbes was counted using the plate-count method and stated in CFU/mL.

2.2.7. The Determination of the Bacterial Growth Phase

The determination of the growth phase was important to determine the growth curve in order to determine the speed of which the bacteria reached the exponential phase and the bacteria generation time. Culture preparation was done by inoculating 0.1 ml of the bacterial isolate to 10 ml SWC-broth medium and incubating it for 24 hours at 29^{o}C. Numbers were counted every 2 hours using plate count method. The cultures were incubated at 29^{o}C for 24 hours. The population of microbes growing was stated in the number of colony forming units (CFU) and was calculated using the following equation:

$$PM = \frac{K}{A \times B \times C}$$

Where:

PM = the population of microbes (CFU/mL)

K = the number of colonies

A = the volume inoculated to the dilutant medium (mL)

B = the dilution stage where the microbe colonies were counted

C = the volume of the diluted medium which was inoculated to the solid medium (mL)

2.3. Feed preparation

The experimental feed used was commercial feed (*Otohime Marine Weaning Diet EP 1 Japan*) size 1.5 mm. The feed was weighed 3 %/BW then mixed with 1% based on the respective treatments (A: probiotic RM3, B: probiotic RM4, C: probiotic RM5, D: probiotic RM7) and 2% egg white by spraying the mixture using a syringe and mixing thoroughly. The control feed was only mixed with 2% egg white. The fish were kept for 40 days and fed 3 %/BW. The feeding frequency was twice a day, at 08.00 and 16.00.

2.4. The in vivo testing of the probiotic bacteria candidate in humpback groupers

The test animals used in this research were humpback groupers (4.65±0.44 g), obtained from Balai Budidaya Air Payau (The Brackish Water Cultivation Station- BBAP) Situbondo, and came from the same parents. Before subjected to the tests, the fish were adapted for 10 days in 1 m^3 fiberglass holding tanks equipped with an aeration system installed in the wet lab in the Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. The experiments were done in 60x30x30 cm^3 aquariums with a water volume of 36 L each. Aeration was provided for every aquarium. Each aquarium held 5 test fish. Before the experiment, the fish were not fed for 24 hours. Siphoning was done after feeding to dispose of feces and any leftover feed. Every day, 10% of the water was replaced.

2.4.1. Observation parameters

The parameters observed in this study were protein and fat retention, daily growth rate, feed efficiency, survival rate, and digestive enzyme activity.

Nutrient (protein, fat) retention

The nutrient retention value was calculated using the equation in [30]:

RN = [(F-I)/P] x 100%

Note:

RN = Nutrient retention: protein and fat (%)

F = The amount of nutrients in the fish's body at the end of the keeping period (gram)

I = The amount of nutrients in the fish's body at the beginning of the keeping period (gram)

P = The amount of nutrients consumed by the fish (gram)

Daily Growth Rate (DGR)

The DGR was calculated using the equation in [31]:

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

Note:

We = The fish's weight at the end of the treatment (gram)

Ws = The fish's weight at the beginning of the keeping period (gram)

d = Keeping period (days)

Feed Conversion Rate (FCR)

Feed efficiency was calculated using the equation in [32]:

$$\text{FCR} = \frac{feed\ given\ (dry\ weight)}{weight\ gain\ (wet\ gain)}$$

Survival rate

The survival rate was calculated using the following equation:

Survival = [the number of fish at the end of the keeping period/the number of fish at the beginning of the kepping period] x 100%

Digestive Enzyme Activity Analysis

This analysis was done at the end of the keeping period. The digestive tracts of 2 fish were taken in each replicate and weighed. The crude enzyme extract filtrate was taken for amylase, lipase and protease enzyme activity using a method similar to the enzyme activity testing for the bacteria (see screening method for enzyme activity).

3. Results And Discussion

3.1. The isolation and selection of probiotic bacteria

Fifty-eight isolates were successfully isolated from the humpback grouper's digestive tract. Each bacterial isolate was tested for its ability to hydrolyze starch, casein, and fat. Based on the selection results, 9 isolate which had the strongest hydrolyzing capacity in each medium and had good survival rates were selected. The results of the 9 bacterial isolates' starch, casein and fat hydrolyzing tests (Figure 1). Probiotic bacteria isolated in this study were the bacteria which showed amylolytic, proteolytic, and lipolytic activities. The results of this study were in line with the study by [22] who showed that a large number of bacteria which produced enzymes such as amylolytic, proteolytic, and cellulolytic enzymes were found in the *Labeo rohita* and *Channa punctatus* fish's gastrointestinal tract. Similar results were obtained by [23] in *Hippocampus kuda,* and [33] for screening marine *Streptomyces* spp.

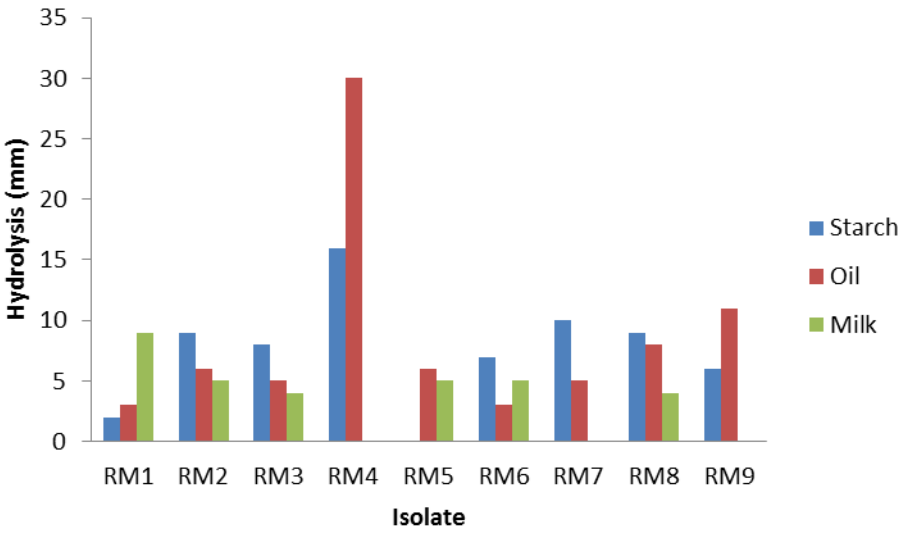

Figure 1. Amylolytic, proteolytic, and lipolytic activities of probiotic bacteria candidate

Based on the results of the selection through amylase, protease, and lipase enzyme activity testing, 6 isolates which showed the strongest enzyme activity were chosen, i.e. isolates RM2, RM3, RM4, RM5, RM7, and RM8 (Table 1). The bacterial isolates which were selected based on amylase activity were RM3, RM5, RM4, RM7, and RM2. The bacterial isolates chosen based on protease activity were isolates RM8, RM7, RM4, RM3, and RM2. The lipase enzyme activity in each isolate showed similar results, ranging between 0.06 ±0.002 and 0.07 ±0.003 U/mL/min. In increasing the feed's nutritional value, probiotics are able to produce several exogenous enzymes to digest feed such as amylase, protease, lipase and cellulase [34, 35]. These exogenous enzymes will help the host's endogenous enzymes in hydrolyzing feed nutrients such as breaking down long chains found in carbohydrates, protein and fat in the feed. Breaking down complex molecules into simpler molecules will make the process of digesting and absorbing in the fish's digestive tract easier.

Table 1. Enzyme activity of candidate probiotic bacteria

Isolate code	Enzyme Activity		
	Amylase	Protease	Lypase
RM1	0.394 ±0.07	0.0049 ±0.00011470	0.06 ±0.002
RM2	0.468 ± 0.10	0.0084 ±0.00012700	0.07 ±0.002
RM3	0.774 ±0.28	0.0094 ±0.00140100	0.07 ±0.002
RM4	0.569 ±0.02	0.0100 ±0.00388600	0.07 ±0.003
RM5	0.690 ±0.19	0.0078 ±0.00299400	0.07 ±0.002
RM6	0.204 ±0.09	0.0076 ±0.00095600	0.07 ±0.002
RM7	0.541 ±0.28	0.0143 ±0.00541500	0.07 ±0.002
RM8	0.329 ±0.16	0.0156 ±0.01102100	0.07 ±0.002
RM9	0.397 ±0.09	0.0036 ±0.00197500	0.07 ±0.002

The six probiotic bacteria candidates chosen were re-selected based on their antagonistic ability towards pathogens, their tolerance to acid and base conditions, their ability to adhere and form biofilm, their pathogenic properties towards the host, and their growth based on their growth rates and the length of their steady state at the peak of the population. The probiotic bacteria candidates' ability to produce antimicrobial substances is a crucial criterion; the bacteria are expected to be able to suppress the growth of pathogenic bacteria in the humpback grouper's digestive tract. The selected probiotic bacteria candidates's antagonistic abilities are presented in Figure 2. Based on the results, it was observed that the strongest antagonistic activity towards the bacteria *V. alginolyticus*^{rif} was shown by isolate RM7 at 2.30 x 10^3 (log CFU/mL), followed by isolates RM4, RM3, RM5, RM2 and RM8.

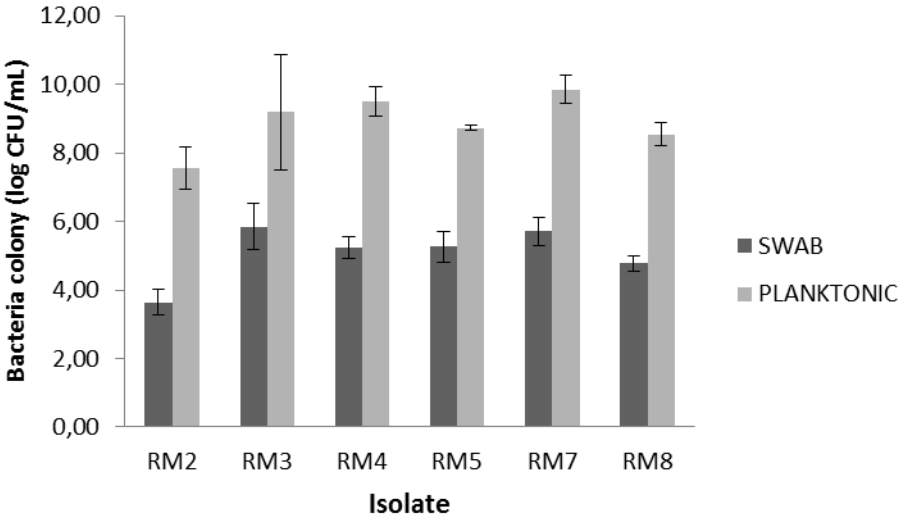

Figure 2. Ability to inhibit the growth of fish pathogen *V.alginolyticus*^{rif} (log CFU/mL)

The pathogenicity test was done to discover whether the bacterial isolates obtained were pathogenic or not. The results of the testing after being kept for 10 days showed that the highest survival rates were shown by isolates RM 2, RM 3, RM 4, RM5 and RM 7, respectively, at 100%. The survival rate of the negative control was 100% and the positive control 33.33 ± 11.55%.

Based on the resistance to acid and base test, it was observed that all the probiotic bacteria candidates showed a good tolerance to acid and base and were able to proliferate in acidic (pH 2.5) and alkaline (pH 8.5) conditions. During the 8 hour observation period, the smallest log difference was shown by RM4, both at pH 2.5 and pH 8.5, followed by RM5, RM7, and RM3 (Figure 3). This indicates that the bacteria are able to survive in the stomach which has a low pH due to the secretion of stomach acids and are also able to withstand bile salts which have a high pH. These ablilities are suggested to be caused by the fact that the isolates normal micro flora normally found in the digestive tract which have adapted to the acidic condition of the stomach and the bile salts in the intestinal tract.

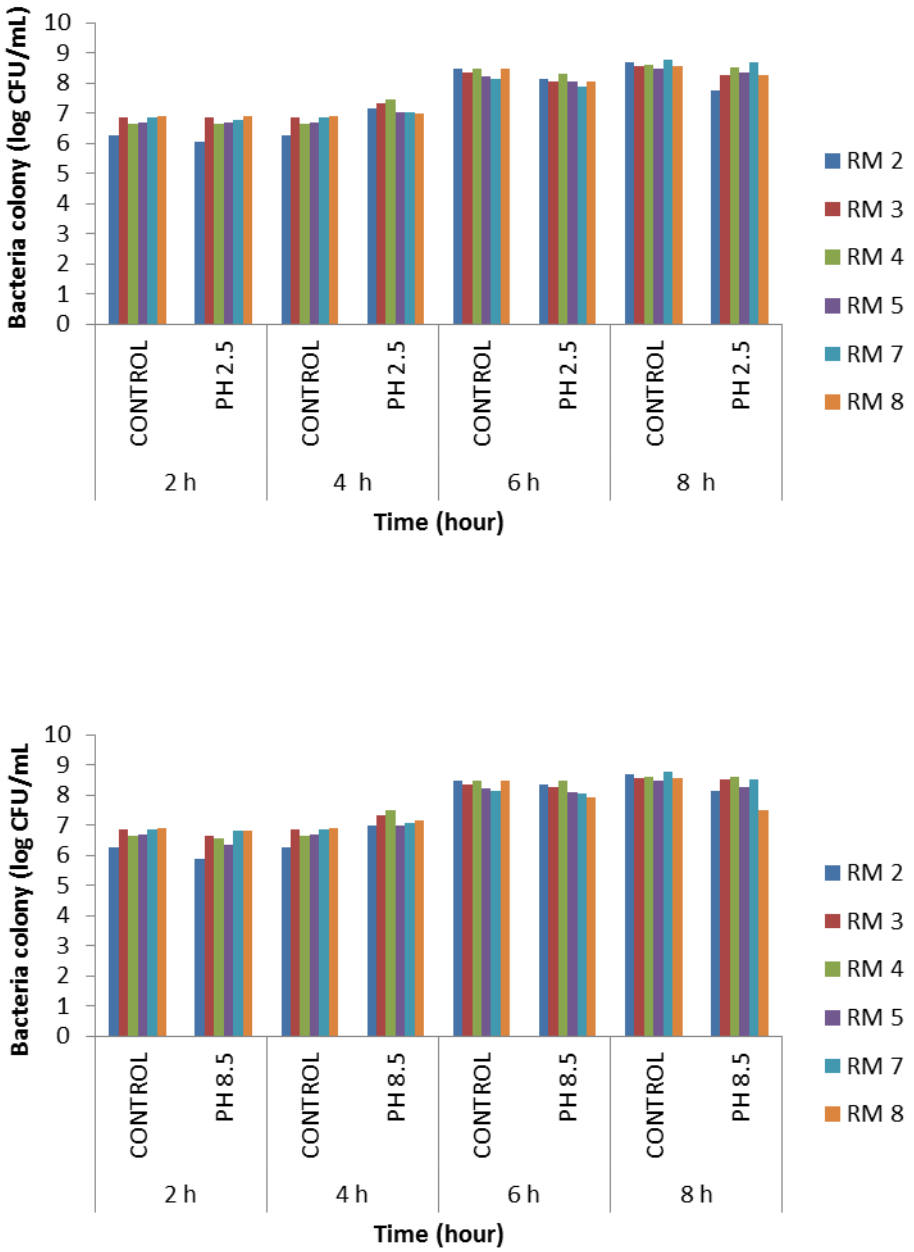

Figure 3. Population of candidate probiotic bacteria in media with pH 2.5 and pH 8.5

The ability to form biofilm is determined by the adhesion factor on solid surfaces or substrates. This test was a simulation of the bacteria's ability to adhere to the surface of the intestinal lumen. All the probiotic candidates showed an ability to adhere and form a biofilm on the surface of the stainless steel plate. The isolate RM3 showed the best ability at 1.16 x 10^6 CFU/mL, followed by RM7, RM5, RM4, RM8 and RM2 (Figure 4).

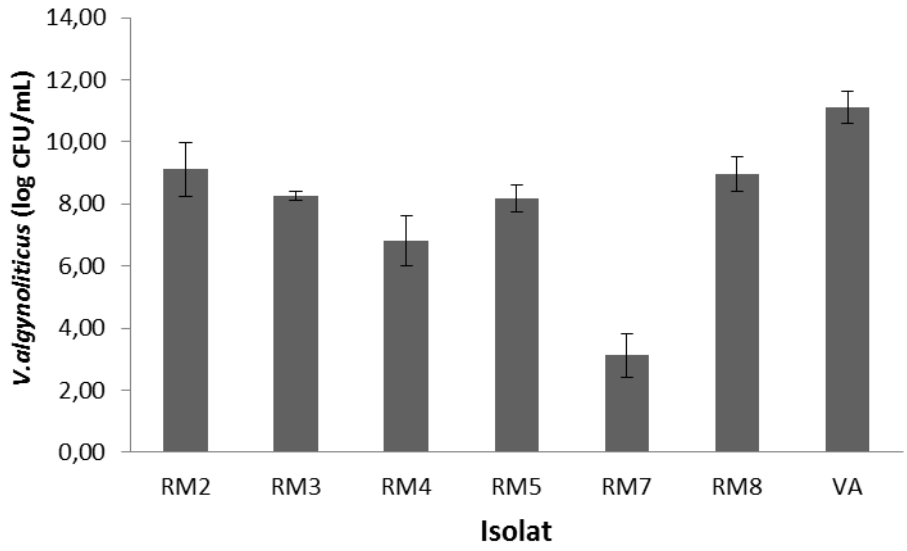

Figure 4. Ability of probiotic bacteria candidate to form biofilm (log CFU/cm2).

The bacterial isolates' growth was measured to determine the growth phases of each chosen bacterial isolate. This is related to the best time to harvest the cells to produce a product or metabolic compound such as enzymes, antimicrobes, vitamins, organic acids, fatty acids, amino acids, and peptides. The growth curves formed in the observation period of 24 hours showed that each bacterial isolate had varied patterns. Based on the initial growth phase or lag phase during the counting of colony numbers, it was discovered that the probiotic bacteria candidates RM4 and RM 7 had the longest maximum time or in other words they could reach their exponential phase fastest compared to the other probiotic bacteria candidate isolates. Identification result with API 20E showed that RM 3 significantly 99.3% with *Ewingella americana*, RM 4 significantly 86.0 % with *Vibrio alginolyticus*, RM 7 significantly 96.9 % with *Pseudomonas flourescens* and API 20NE for RM5 significantly 99.4% with *Sphingomonas paucimobilis*.

3.2. Growth Performance

Through this screening, 4 probiotic candidates were obtained and were then used in the *in vivo* test with humpback groupers. Based on the study results, it was discovered that the fish fed with feed containing the probiotic bacteria strains RM3, RM4, and RM 5 showed higher protein retention and fat retention ($P<0.05$), higher daily growth rates ($P<0.05$), and lower feed conversion ratio (FCR) ($P<0.05$) than those of the control. The treatment RM7 was not significantly different from the control ($P>0.05$; Table 2). The *in vivo* testing showed that the addition of probiotic in feed resulted in better growth performance than the control . Similar results were found in other studies which found that the addition of probiotics to feed could improve weight gain, the specific growth rate, and the feed conversion ratio in *Penaeus vannamei* [15], rainbow trout (*Onchorhynchus mykiss*) [36] and *Labeo rohita* fish [37].

The high growth performance shown in the probiotic treatments was strongly related to the high enzyme activity in the fish's digestive tract. The result of the enzyme activity analysis in this study showed that the enzyme amylase was detected in higher quantities compared to the enzymes protease and lipase. It is suggested that the probiotic candidate contributed in producing high levels of extracellular amylase enzyme. The highest results were shown by the probiotic candidates RM5, RM3, and RM 4, at 1.68 ± 0.04 U/mL/min, 1.21 ± 0.08 U/mL/min, and 1.06 ± 0.01 U/mL/min, repsectively. Different probiotics show different effects on enzyme activity. The enzyme activity of protease, amylase and lipase in *Cyprinus carpio* which had been supplemented with the probiotic *Bacillus* sp showed higher results than photosynthetic bacteria ; however, supplementation using the combination between the two probiotics showed the highest digestive enzyme activity [38]. Similar results were reported by [15] for *Penaeus vannamei.* The action and positive effects of probiotics in fish cultivation are the due to the ability to improve the host species' nutrition through the production of supplemental digestive enzymes and a high growth and feed efficiency, to protect against intestinal disorders and to absorb anti-nutritional factors in the feed [4; 14; 37].

Table 2. Growth performance and digestive enzyme activity of C. altivelis fed different supplementation of probiotics

Parameter	Treatments				
	Control	RM3	RM4	RM5	RM7
Weight gain (g)	9.87 ± 2.23^{b}	20.81 ± 4.12^{a}	20.17 ± 1.22^{a}	20.22 ± 3.62^{a}	12.14 ± 3.46^{b}
Protein retention (%)	18.84 ± 0.18^{b}	30.22 ± 2.15^{a}	32.19 ± 2.32^{a}	28.17 ± 5.89^{a}	17.89 ± 3.61^{b}
Lipid retention (%)	21.09 ± 2.25^{b}	32.47 ± 1.41^{a}	32.68 ± 5.46^{a}	32.63 ± 0.62^{a}	23.75 ± 1.68^{b}
DGR (%)	0.81 ± 0.16^{b}	1.47 ± 0.23^{a}	1.47 ± 0.23^{a}	1.48 ± 0.36^{a}	0.92 ± 0.20^{b}
FCR	3.31 ± 0.68^{a}	1.73 ± 0.22^{b}	1.77 ± 0.12^{b}	1.68 ± 0.28^{b}	2.83 ± 0.59^{a}
Survival rate (%)	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
Protease (U/mL/menit)	0.07 ± 0.006^{c}	0.10 ± 0.007^{b}	0.13 ± 0.006^{a}	0.07 ± 0.001^{c}	0.10 ± 0.002^{b}
Lipase (U/mL/menit)	0.05 ± 0.002^{b}	0.05 ± 0.003^{a}	0.05 ± 0.001^{a}	0.05 ± 0.001^{b}	0.05 ± 0.001^{b}
Amilase (U/mL/menit)	0.85 ± 0.04^{e}	1.21 ± 0.08^{b}	1.06 ± 0.01^{c}	1.68 ± 0.04^{a}	0.98 ± 0.01^{d}

Data expressed as Mean ± SD

Mean values in some row with different superscript vary significantly ($P<0.05$)

The increased digestive enzyme activity in this study could explain why the addition of probiotics could improve the humpback grouper's ability to digest feed components such as protein, starch, and fat. Some feed ingredients which are broken down into simpler molecules and then absorbed by the intestines, enter the blood flow, and are distributed to all tissues and entered cells. In cells, glucose is oxidized to produce energy [39]. Protein and fat are retained in the tissues, improving growth performance. In this study, the increased enzyme activity was followed by the increase of SGR, protein retention and fat retention, and the improved FCR value in the probiotic RM3, RM4, and RM5 treatment.

4. Conclusions

Selection of probiotic candidate resulted 4 bacterial isolates, i.e. RM3, RM4, RM5 and RM 7 strain. Identification result showed that RM 3 significantly 99.3% with *Ewingella americana*, RM 4 significantly 86.0 % with *Vibrio alginolyticus*, RM 7 significantly 96.9 % with *Pseudomonas flourescens* and RM5 significantly 99.4% with *Sphingomonas paucimobilis*. The application test of probiotic bacteria candidate in humpback grouper (*C.altivelis*) showed that increased enzyme activity was followed by the increase of SGR, protein retention and fat retention, and the improved FCR value in the probiotic RM3, RM4, and RM5 treatment except RM7 than control.

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