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Original research article

Effect of Probiotic *Bacillus megaterium* PTB 1.4 on the Population of Intestinal Microflora, Digestive Enzyme Activity and the Growth of Catfish (*Clarias* sp.)

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ABSTRACT

This study aimed to analyze the effect of *Bacillus megaterium* PTB 1.4 on the population of intestinal microflora, digestive enzyme activity, and the growth of catfish. Gnotobiotic and normal fish were used. Treatment using gnotobiotic was divided into gnoto (with feed and 100 μg/mL rifampicin) and gnotoplus (with feed, 100 μg/mL rifampicin, and 1% probiotic); whereas treatment using normal fish was divided into normalplus (with feed and 1% probiotic) and normal (only feed). The amount of bacteria on gastrointestinal tract was measured 30 days after treatments using the total plate count method. The results indicated no significant difference in bacterial growth between gnotobiotic and normal fish. The total amount of probiotic bacteria with normalplus treatment was significantly different with gnotoplus. The activity of protease and amylase enzymes, and specific growth rate in normalplus treatment were significantly higher ($p < 0.05$) than other treatments. *Bacillus megaterium* PTB 1.4 increased the activity of digestive enzymes and the growth of catfish.

1. Introduction

As human population grows, needs for animal protein also increase. Consuming fish is one of the ways to meet human needs for animal protein and catfish is an aquaculture product which has relatively high protein. High demand for catfish increases intensive catfish aquaculture production. However, various problems are emerging related to the intensive fish aquaculture activities, such as low feed digestibility, increasing disease and decreasing water quality. The use of artificial feed in intensive farming caused lower feed digestibility. It was because the content of feed material in artificial feed is difficult to digest compared with live feed.

Feed digestibility in aquaculture highly affects fish growth and production cost. It can be improved by increasing the activity of digestive enzymes capable of breaking feed nutrients down, one of which uses probiotic bacteria. The benefits the probiotic bacteria bring for the host are among others as a nutrient source and contribution to enzymatic digestion process (Balcazar *et al.* 2006). Some studies indicated that probiotic bacteria are capable of increasing digestive enzymes and the growth of *Fenneropenaeus indicus* (Ziaei-Nejad *et al.* 2006), *Litopenaeus vannamei* (Wang 2007; Zokaeifar *et al.* 2012), *Cromileptes altivelis* (Marlida *et al.* 2014), and abalone *Haliotis asinina* (Faturrahman *et al.* 2015), increasing the growth rate and feed efficiency of grouper (Sun *et al.* 2010), improving nutrient digestibility (Putra and Widanarni 2015), growth (Utami *et al.* 2015), and enhancing immune response in tilapia (*Oreochromis* sp.) (Aly *et al.* 2008).

Hamtini *et al.* (2015) successfully isolated bacteria from catfish digestive tract and obtained PTB 1.4 isolate that has proteolytic and amylolytic activities. Based on the results of total suspended solids, isolate PTB 1.4 is capable of degrading feed. Isolate PTB 1.4 was identified as *Bacillus megaterium* (Hamtini *et al.* 2015). The purpose of this study was to analyze the effect of probiotic bacteria *B. megaterium* PTB 1.4 on the population of intestinal microflora, digestive enzyme activity, and the growth of catfish.

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2. Materials and Methods

2.1. Mutation of rifampicin resistance

Rifampicin-resistant mutant bacteria was obtained by growing 10^{10} CFU/mL of probiotic bacteria PTB 1.4 on Tryptic Soy Agar (TSA) added with 50 μg/mL rifampicin. Grown bacteria were re-cultured on TSA media added with 100 μg/mL rifampicin before evaluation of the activity of proteolytic and amylolytic enzymes and the growth rate of the bacteria obtained.

2.2. Proteolytic and amylolytic activity tests

Rifampicin-resistant bacteria were grown on TSA medium with 1% skim milk and 1% starch for proteolytic and amylolytic tests, respectively. The activity of the enzymes can be identified through the formation of clear zone around the isolate. Proteolytic and amylolytic indices were measured using the following equation (Lim *et al.* 1987).

$$IP/IA = \frac{X1 - X2}{X2}$$

where,

IP/IA = index of proteolytic/amylolytic activity
$X1$ = average diameter of clear zone
$X2$ = average diameter of colony

2.3. Bacterial growth

Observations were conducted to determine the stationary phase of the bacterial growth as the phase is the basis of cell harvesting for the application of probiotic. Pure PTB 1.4 Rf^R inocula (rifampicin-resistant) were inoculated on 20 mL Tryptic Soy Broth (TSB) and incubated at room temperature for 24 hours before re-inoculation of 10 mL culture on 90 mL TSB medium and incubation at 28°C and 120 rpm for 24 hours. The bacterial growth was measured using total plate count and turbidity method.

2.4. Pathogenicity test

A total of 0.1 mL of each probiotic bacteria PTB 1.4 wild type, probiotic bacteria PTB 1.4 Rf^R (rifampicin-resistant), 10^8 CFU/mL *Aeromonas hydrophila* as positive control, and phosphate buffer saline as a negative control was injected into catfish intramuscularly. Catfish were reared in $60 \times 30 \times 30$ cm^3 aquarium at a density of 10 individuals per aquarium and with average weight of 5.57 ± 0.52 g. The fish were reared for 14 days and the mortality was observed.

2.5. Probiotic viability test in feed

Probiotic bacteria of 10^8 CFU/mL in concentration were grown on TSB medium. Suspension was centrifuged at 5000 rpm for 30 minutes and phosphate buffer saline was added to the pellet before re-suspension. A total of 1 mL probiotic bacteria was added to the test feed and viability test of probiotic in test feed was carried out on gnotoplus and normalplus feeds. Gnotoplus feed contained 1% probiotic and 100 μg/mL rifampicin, whereas normalplus contained 1% probiotic. All test feeds were coated with 2% egg whites.

A total of 1 g test feed was diluted into 9 mL 0.85% NaCl before serial dilution up to 10^{-6}. The dilution result was spread on TSA medium with 100 μg/mL rifampicin, after 24 hours incubation at 28°C the colonies were counted. Probiotic viability in feed was observed on 1, 5, 10 days after storage at 4°C.

2.6. Bioassay of probiotic bacteria in catfish

2.6.1. Feed preparation

Commercial feed with 30% protein was used in this experiment. The feed was sterilized at 120°C for 15 minutes for reducing contaminant. The density of probiotic bacteria used was 10^{10} CFU/mL. There were four types of feed tested, i.e. gnoto (with 100 μg/mL rifampicin), gnotoplus (with 100 μg/mL rifampicin and 1% probiotic), normalplus (with 1% probiotic), and normal (without rifampicin and probiotic). The binder used 2% egg whites.

2.6.2. Fish experiment and gnotobiotic catfish

Catfish used in this experiment were divided into normal (without any antibiotic treatment) and gnotobiotic organism (with antibiotic treatment). Gnotobiotic organism is catfish that had no bacteria on its body and digestive tract. Gnotobiotic catfish treatment was carried out by adding antibiotics (250 mg/L ampicillin, 125 mg/L rifampicin and 250 mg/L chloramphenicol) into aquarium water. The catfish were then starved for 24 hours and feed containing 100 mg/L rifampicin was given for 4 days. After antibiotic treatment, the aquarium water was replaced with new water.

2.6.3. Growth test

Catfish used in this experiment were reared in $60 \times 30 \times 30$ cm^3 aquarium at a density of 15 individuals per aquarium and with average weight of 11.41±0.23 g. The growth test was carried out with four treatments of three replications each. The treatment consisted of gnoto (gnotobiotic catfish with feed containing 100 μg/mL rifampicin), gnotoplus (gnotobiotic catfish with feed containing 100 μg/mL rifampicin and probiotic 1%), normalplus (normal catfish with feed containing 1% probiotic) and normal (normal catfish without any treatment). Fish were grown for 30 days and the feed was given three times a day by *at satiation*. To maintain water quality, 50% water capacity in the aquarium was replaced every 3 days.

2.6.4. Enumeration of intestinal bacterial population

A total of two fish were randomly selected from each treatment. The fish were starved for 20 hours before sampling. The digestive tract of the fish was aseptically taken out from each fish. The intestine was weighed to make 1 g sample. Sample was transferred into a tube containing 9 mL 0.85% sterile NaCl and homogenized before serial dilution up to 10^{-6}. Total bacterial counts were determined by plating on TSA medium, whereas total probiotic bacteria on TSA with 100 μg/mL rifampicin.

2.6.5. Digestive tract enzyme activity

Digestive tract of 1 g was homogenized in 5 mL 0.05 M phosphate buffer pH 7.5 and centrifuged at 6000 rpm for 30 minutes at 4°C. The supernatant was assayed to measure the enzyme activity. Protease activity was assayed following Walter (1984), i.e. using casein as the substrate and reacting it with Folin reagent. Amylase activity was assayed following Bernfeld (1955), i.e. using starch as the substrate and reacting it with 3.5-dinitrosalicylic acid.

2.6.6. Digestibility analysis

After 30 days, the fish were reared in aquarium at a density of 10 individuals per aquarium for 15 days. Feed treatment added with 0.6% chromium (Cr_2O_3) was given to the fish. Feces were collected by siphoning the feces after feeding and stored at 4°C for subsequent analysis. Pooled feces of each treatment were dried in oven at 110°C for 4–6 hours. Total digestibility and protein digestibility were calculated following Takeuchi (1988), i.e. using equations: total digestibility = 100 − (% Cr_2O_3 in feed/% Cr_2O_3 in feces × 100); and protein digestibility = 100 − [(% Cr_2O_3 in feed/% Cr_2O_3 in feces × 100) × (% protein in feces/% protein in feed) × 100].

2.6.7. Measurement of growth parameters

Growth parameters measured were survival rate (SR) and specific growth rate (SGR) following Huisman (1987), in addition to feed conversion ratio (FCR) with the following equation (Zonneveld *et al.* 1991):

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

where,

Nt = number of fish at the end of the rearing period (individual); and
No = number of fish at the beginning of the rearing period (individual)

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

where,

wt = weight of fish at the end of the rearing period (g);
wo = weight of fish at the beginning of the rearing period (g); and
t = duration of the rearing period (day)

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

where,

F = total weight of feed intake (g);
Wt = weight of fish at the end of the rearing period (g);
Wo = weight of fish at the beginning of the rearing period (g); and
D = weight of dead fish during rearing period (g)

2.6.8. Statistical analysis

A complete random design with four treatments and three replications was used. All data means were compared using Duncan multiple range tests with SPSS 21 program. A significance level of $p < 0.05$ was used for all test, and data were reported as means ± standard errors.

3. Results

3.1. Mutation of rifampicin resistance

The proteolytic and amylolytic indices of isolate PTB 1.4 Rf^R (rifampicin-resistant mutants) were 1.07 and 0.58, respectively. The stationary phase of isolate PTB 1.4 Rf^R was 12 hours, resulting in the phase period was used as the basis of harvesting cells time for probiotics application (Figure 1).

3.2. Pathogenicity test

The result of pathogenicity test of probiotic bacteria in catfish indicated that PTB 1.4 and PTB 1.4 Rf^R were not pathogenic, following the results of the SR observed during the 14 rearing days in which the SR of PTB1.4 (wild type), PTB 1.4 Rf^R, and negative control treatments were 100%, whereas the SR of the positive control treatment was 3.3% (Table 1).

3.3. Probiotic viability test in feed

The results of bacterial viability test in feed during the observation indicated that probiotic bacteria added to the feed can survive with cell density of 10^6 CFU/mL. Probiotics in gnotoplus and normalplus feeds showed no significant difference (Table 2).

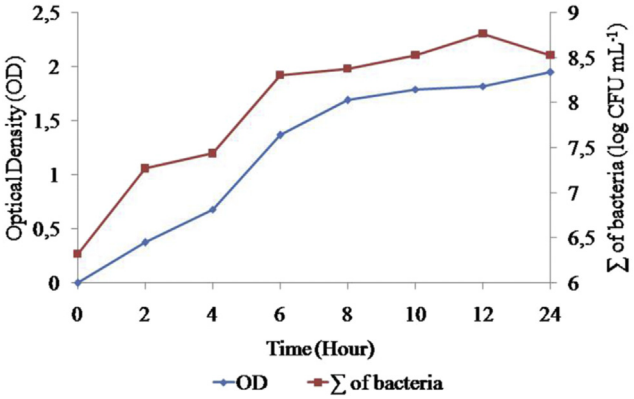

Figure 1. Growth curve of bacteria isolate PTB 1.4 Rf^R.

Table 1. The pathogenicity test of probiotic bacteria on catfish

Treatments	Survival rate (%)
Negative control	100.0
Positive control	3.3
PTB 1.4	100.0
PTB 1.4 Rf^R	100.0

3.4. Population of intestinal microflora

Total bacteria found in the digestive tract of catfish after being treated for 30 days showed a difference in the total number of bacteria. However, there was no significant difference between gnoto ($2.24 \pm 0.41 \times 10^5$ CFU/g) and gnotoplus treatments ($5.90 \pm 2.32 \times 10^5$ CFU/g); and between normalplus ($32.90 \pm 4.74 \times 10^5$ CFU/g) and normal treatments ($36.33 \pm 7.51 \times 10^5$ CFU/g). The total number of probiotic bacteria in gnotoplus ($3.29 \pm 0.78 \times 10^5$ CFU/g) and normalplus ($5.99 \pm 0.57 \times 10^5$ CFU/g) treatment were significantly different (Table 3).

3.5. Digestive tract enzyme activity

The activity of protease was significantly higher ($p < 0.05$) than in normalplus treatment (1.32 ± 0.09 IU/g digestive tract), followed with gnotoplus treatment (0.96 ± 0.06 IU/g digestive tract) gnoto and normal treatments (Figure 2A). The same result was found for the activity of amylase, where the highest enzyme activity was found in normalplus treatment (0.35 ± 0.02 IU/g digestive tract; $p < 0.05$; Figure 2b).

3.6. Digestibility analysis

The total digestibility was significantly higher ($p < 0.05$) in normalplus treatment (76.90 ± 0.98%) than gnoto and normal treatments. However, the value was not significantly different with gnotoplus treatment (74.43 ± 1.17%). In addition, the result of protein digestibility test indicated that normalplus treatment (93.22 ± 0.99%) was significantly higher ($p < 0.05$) than any other treatments (Table 4).

Table 2. The bacteria viability test on PTB 1.4 Rf^R in feed after storage

Treatment of feed	Total probiotic bacteria in feed (CFU/g) at day		
	1	5	10
Gnotoplus test feed	8.4×10^6	2.3×10^6	1.1×10^6
Normalplus test feed	1.2×10^7	9.4×10^6	1.1×10^6

Table 3. Total bacteria and probiotic bacteria in the digestive tract of catfish

Treatment	Total bacteria (10^5 CFU/g)	Probiotic bacteria (10^5 CFU/g)
Gnoto	2.24 ± 0.41^a	–
Gnotoplus	5.90 ± 2.32^a	3.29 ± 0.78^a
Normalplus	32.90 ± 4.74^b	5.99 ± 0.57^b
Normal	36.33 ± 7.51^b	–

Mean values in the same column with a different superscript are significantly different ($p < 0.05$).

Table 4. Total of digestion (Td) and protein digestion (Pd) in the catfish given by feed treatment

Parameter	Treatment			
	Gnoto	Gnotoplus	Normalplus	Normal
Td (%)	71.61 ± 1.83^a	74.43 ± 1.17^{ab}	76.90 ± 0.98^b	73.91 ± 2.42^a
Pd (%)	86.99 ± 2.53^a	88.71 ± 2.41^a	93.22 ± 0.99^b	85.28 ± 1.51^a

Mean values in the same column with a different superscript are significantly different ($p < 0.05$).

Table 5. Survival rate (SR), specific growth rate (SGR) and feed conversion ratio (FCR) of fish

Parameter	Treatment			
	Gnoto	Gnotoplus	Normalplus	Normal
SR (%)	100 ± 0.00^a	100 ± 0.00^a	100 ± 0.00^a	100 ± 0.00^a
SGR (%)	1.92 ± 0.23^a	2.11 ± 0.19^a	2.69 ± 0.36^b	1.98 ± 0.23^a
FCR	1.77 ± 0.12^a	1.66 ± 0.15^a	1.05 ± 0.11^b	1.49 ± 0.23^a

Mean values in the same row with a different superscript are significantly different ($p < 0.05$).

3.7. Growth parameters

After 30 days, the SRs of all treatments showed no significant difference and the SR was 100 ± 0.00% in all treatment. The application of probiotic in feed significantly increased SGR where normalplus (2.69 ± 0.36%) was higher than other treatments. The FCR in normalplus treatment (1.05 ± 0.11) was the lowest, followed by gnotoplus, normal and gnoto treatments, respectively. FCR in normalplus treatment was significantly lower ($p < 0.05$) than other treatments (Table 5).

4. Discussion

Rifampicin-resistant mutant isolate PTB 1.4 showed to have proteolytic and amylolytic activities, indicated with clear zone formations in TSA medium containing skim milk. The clear zone suggested that isolate PTB 1.4 Rf^R was capable of producing protease enzyme to degrade protein in skim milk. Amylase test also indicated the same result, in which clear zone was formed on medium containing starch, suggesting that isolate PTB 1.4 Rf^R is capable of producing amylase enzyme to degrade starch in the media. Isolate PTB 1.4 is *B. megaterium*, where *Bacillus* spp. group is known to have ability to produce extracellular enzymes (such as protease and amylase) (Moriarty 1998). The values of proteolytic (0.6) and amylolytic (0.61) indices of wild type isolate PTB 1.4 (Hamtini *et al.* 2015) were not significantly different with isolate PTB 1.4 Rf^R, suggesting that mutant isolate PTB 1.4 (PTB 1.4 Rf^R) still preserves its wild type's proteolytic and amylolytic activities.

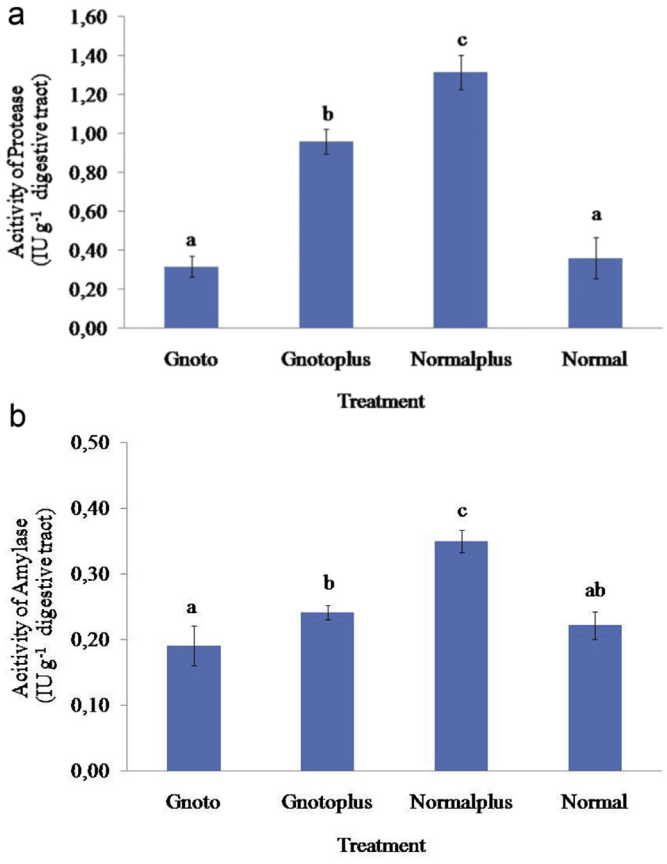

Figure 2. Enzyme activity of (a) protease and (b) amylase in the digestive tract of catfish after 30 rearing days. Means with the different superscript are significantly different ($p < 0.05$).

Pathogenicity test of the probiotic bacteria in wild type PTB 1.4 and PTB 1.4Rf^R indicated that the bacteria were not pathogenic for catfish, proven by their 100% SRs. Probiotic viability test in feed aimed to determine the bacteria's ability to survive in the test feeds. The result showed that probiotic bacteria are capable of surviving in the feed up to 10 days after storage at 4°C with density reached to 10^6 CFU/g feed.

The total numbers of bacteria in the digestive tract after feed with probiotics given in normalplus treatments were lower than normal treatment. The same results were found in Bagheri *et al.* (2008) and Mohapatra *et al.* (2012), where total number of bacteria in the digestive tract of fish decreased after probiotic was added in feed. Probiotics have an action mechanism to suppress microflora populations, i.e. production of antimicrobial compounds, competition of nutrients or adhesion to intestinal wall (Verschuere *et al.* 2000). The numbers of probiotic bacteria in digestive tract in gnotoplus and normalplus treatments were significantly different. The amount of probiotic bacteria in digestive tract indicated that probiotic bacteria PTB 1.4 is capable of colonizing fish gastrointestinal tract, where one of the requirements of probiotic bacteria is capability of colonizing the digestive tract of the host (Balcazar *et al.* 2006).

Probiotic bacteria are capable of producing digestive enzymes that help fish use feed nutrients and digest (Bairagi *et al.* 2002). The activity of enzymes in fish digestive tract in normalplus treatment was the highest, allegedly because PTB 1.4 increased digestive enzymes. Similarly, in gnotoplus treatment where PTB 1.4 is able to increase the activity of digestive enzyme compared with gnoto treatment. Exogenous enzymes produced by probiotic bacteria give only a small contribution to total enzyme activity in digestive tract (Ziaei-Nejad *et al.* 2006; Zhang *et al.* 2010). Therefore, such high enzyme activity in gastrointestinal tract was alleged because the probiotic bacteria stimulated the synthesis of endogenous digestive enzymes produced by fish. Generally, endogenous enzyme can be produced by fish, but the presence of probiotics can improve digestive enzyme. Probiotics improve digestive enzyme activity by stimulating the synthesis of endogenous enzyme in the digestive tract (Mohapatra *et al.* 2012).

Digestive enzyme activity can help fish degrade nutrients in feed and subsequently increase digestibility and feed efficiency (Cerezuela *et al.* 2011; Widanarni *et al.* 2015). The high enzyme activity in normalplus treatment increases nutrient digestibility value, and the high value of which indicated that the fish are capable of digesting nutrients in feed properly. Total digestibility and protein digestibility values were the highest in normalplus treatment, indicating that probiotics PTB 1.4 is capable of increasing nutrient digestibility to produce or stimulate digestive enzymes. Probiotics are capable of stimulating the synthesis of endogenous digestive enzymes and improving digestibility (Yanbo and Zirong 2006: Mohapatra *et al.* 2012). Similar results were found in humpback grouper (*Cromileptes altivelis*) where the addition of probiotics and prebiotics in their feed can improve protein digestibility and total digestibility at 91.17 ± 1.75% and 2.08 ± 66.92%, respectively (Marlida *et al.* 2014).

High enzyme activity and high feed digestibility were capable of influencing fish growth and feed efficiency. Normalplus treatment showed the highest in growth and feed efficiency. Similar results were found in *Penaeus monodon* that showed higher value of FCR and SGR with the addition of *B. cereus* (Chandran *et al.* 2014). Digestive enzymes help fish degrade and digest the nutrients in feed, making it easier for fish to absorb nutrients in feed. High digestibility help improve growth and feed efficiency in normalplus treatment. Similar results were also found in tilapia that showed improvement in fish digestibility and growth by adding *Bacillus* NP5 in feed (Putra and Widanarni 2015).

In conclusion, application of probiotic bacteria *B. megaterium* PTB 1.4 in feed maintains the balance of intestinal microflora populations, increases the activity of digestive enzyme and growth of catfish. However, further research is needed on the application of probiotic *B. megaterium* PTB 1.4 in dry form to be more efficient in application.

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