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SUPPLEMENTATION OF PROBIOTIC (*Bacillus subtilis*) FOR IMPROVEMENT OF GROWTH PERFORMANCE AND DIGESTIVE ENZYMES ACTIVITY OF *PUNTIUS JAVANICUS* UNDER INTENSIVE AQUACULTURE SYSTEM

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ABSTRACT

The experiment was conducted to examine effects of different doses of *B. subtilis* probiotic supplementation in feed on protein digestibility, growth and activity of digestive enzymes of *P. javanicus* on intensive farming system. *P. javanicus* fingerlings used as test animals. The treatments were *B. subtilis probiotic* on feed. Those were treatments A (0 CFU g^{-1} feed), B (0.75 x 10^7 CFU g^{-1} feed), C (1.50 x 10^7 CFU g^{-1} feed) and D (2.25 x 10^7 CFU g^{-1} feed). Observed parameters were protein digestibility (ADC_P), efficiency of feed utilization (EFU), ratio of feed conversion (FCR), ratio of protein efficiency (PER), relative growth rate (RGR), survival rate (SR), digestive enzymes activity and quality of water. The result showed that supplementation of *B. subtilis* on feeds significantly ($P \leq 0.05$) affected ADC_P, EFU, FCR, PER, RGR, SR and digestive enzyme activities. The experiment was conducted under the viable water quality. The optimum supplementation dose of *B. subtilis* was 1.50 x 107 CFU g^{-1} feed resulted in the highest value of ADC_P (75.28%), EFU (74.06%), FCR (1.52), PER (3.78), RGR (3.23% / day), SR (98.33%) and digestive enzymes (protease and amylase) activities were 4.32, 3.42 U g^{-1} protein, respectively.

Key words: probiotic, *Bacillus subtilis,* growth performance, *Puntius javanicus.*

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INTRODUCTION

The java barb (*Puntius javanicus*) is one of the potential Indonesian native fish and one of the fishery commodities favored by the community. This has led to an increase in java barb production so that market demand can be fulfilled by implementing the intensive fish culture. The success of intensive java barb cultivation is determined by the availability of quality feed. Quality feed is an important factor in intensive aquaculture and it is the largest cost in intensive fish production processes (Tawwab *et al*., 2010), however, quality feed should not only contain nutrients that meet fish requirements, but also make fish resistant to the disease so that it remains healthy and can increase growth (Goda *et al*., 2012). Therefore, various efforts need to be made to improve the quality of feed. One of these efforts is by adding probiotics into the feed.

Probiotics are living microbes when given in sufficient quantities can bring about healthier state of the host and can increase balance of microbes in the digestive tract (Nayak, 2010). Nowadays, the main strategy used is the probiotics supplementation into fish feed that can improve growth (Ghosh *et al*., 2003). Furthermore, Iribarren *et al*., (2012) expressed that the probiotics supplementation into feed can increase growth and efficiency of feed utilization so that it can reduce the accumulation of waste in the water. The probiotics use in fish aquaculture activities to boost growth and increase the ability of resistance to disease has been reported by Nandi *et al*., (2017). The rapid probiotics implementation in fish aquaculture was reported by Gatesoupe, (1999) moreover Rengpipat *et al*. (2000) disclosed that proper use of probiotics in aquaculture increased the number of microbes in the intestine, and increased feed absorption, thereby increasing fish growth rates and decreasing ratio of feed conversion (FCR) (Wang *et al*., 2005). Gatesoupe, (2008) also stated that probiotics can improve the digestion of fish by producing digestive enzymes in the intestine leading to increased growth performance. The addition of probiotics into feed can improve the performance of growth and immune response that has been reported in *Oreochromis niloticus* (Wang *et al*. 2008[a]) and Halibut larvae (Bjornsdottir *et al*., 2010); survival rate and activity of digestive enzyme in *Penaeus vannamei* larvae (Zhou *et al*., 2009); to increase feed conversion, growth and increase weight in salmon (Taoka *et al*., 2006; Bagheri *et al*., 2008; Wang *et al*., 2008[b]).

One of bacteria believed to be able to improve the quality of feed and the fish immune system is *Bacillus subtilis* which is spore-forming bacterium generally apllied as a probiotic. *B. subtilis* can raise survival and growth of cultivated fish (Gomez-Gil *et al*., 2008) and has the ability to secrete protease, lipase and amylase (Wang *et al*., 2008[b]). Some studies on *Bacillus subtilis* supplementation into feed for different species included *Macrobrachium rosenbergii* (Keysami *et al*., 2007), *Litopenaeus vannamei* (Liu *et al*., 2009, 2010; Tseng *et al*., 2009), Labeo rohita (Kumar *et al*., 2006; Nandi *et al*., 2017), *Oncorhynchus myskiss* (Adineh *et al*., 2013), and *Sparus aurata* (Arig *et al*., 2013).

Research on *B. subtilis* probiotic supplementation in java barb feed has not yet been done. Based on this reason, the study was conducted to improve protein digestibility, growth and immune response of java barb. The objectives of the study were to examine probiotic supplementation effects of *B. subtilis* in the feed on protein digestibility, growth and activity of digestive enzymes of java barb on intensive aquaculture system.

MATERIALS AND METHODS

Samples: There were 2,400 samples of Java barb fingerlings with the weight ranging from 3.28 to 3.40 g. The fish were obtained from the Freshwater Hatchery and Cultivation Work Unit, Ngrajek, Magelang, Central Java, Indonesia. The fish fingerling used had the uniformed weight, healthy, and there were no deformities which were then acclimatized for 2 weeks and fasted one day before the study (Rachmawati *et al*., 2017). Before the implementation of the study, java barb fingerlingwere weighed to determine the initial weight. The study has been conducted for 60 days. The weighing process to determine the weight gain of java barb was done every week.

Cultivation Preparation: The dimension of cultivation nets was 1x1x1 m^3. There were 16 pieces which were placed in a 6 x 10 m^2 ground pond with a water level of 1.25 m. Java barb cultivation in this study was carried out intensively with fingerling stocking densities of 150 fish / m^2. For protein digestibility analysis, a cultivation container in the form of a fiber tub with a volume of 500 liters of water was used as a place to accommodate fish feces. Collection of fish feces was done every day a few hours after the fish were fed. Fish feces was collected, then filtered, put into sample bottles and stored in the refrigerator.

***Bacillus subtilis*:** *Bacillus subtilis* was retrieved from the digestive system of *Clarias gariepinus* which was obtained from the Division of Fish Health Management of Freshwater Hatchery and Cultivation Work Unit, Ngrajek, Magelang, Central Java, Indonesia. *B. subtilis* culture was prepared by following the method adopted by Kumar *et al*. (2006) that pure *B. subtilis* culture was inoculated into a 500 mL volume cone tube filled with broth. The bacterium has been incubated for 24 hours at 30^o C in an incubator. Then it was centrifuged at 10,000 g for 20 minutes at 4^o Celcius and the floating waste was removed, while the sediment was diluted in saline based phosphate (PBS; pH 7.2). Furthermore, the measurement of the mixture was done using a technique of spread plate on agar nutrient, was fertilized for 24 hours at 30^o C. The suspension of pure bacteria was reserved at 4^o C and used according to the treatments.

Research Methods and Feed Preparation: The experiment was laid out under completely randomized design. There were 4 treatments and 4 replicates per treatment. The treatment in this study was supplementation of different doses of *B. subtilis* probiotics in the feed. This research was carried out in the Freshwater Hatchery and Cultivation Work Unit, Ngrajek, Magelang, Central Java, Indonesia from March to June 2018.

The feed used was artificial feed with 30% protein content supplemented with probiotics *B. subtilis* by spraying on feed according to the doses of treatment A (0 CFU g^1 feed), B (0.75 x 10^7 CFU g^1 feed), C (1.50 x 10^7 CFU g^1 feed) and D (2.25 x 10^7 CFU g^1 feed) and added 0.5% Cr_2O_3 for protein digestibility analysis. The dose determination of *B. subtilis* probiotics in this study was modified findings by Kumar *et al*., (2006) who reported that *B. subtilis* probiotic doses of 1.5 x 10^7 CFU g^1 feed showed the best growth in *Labeo rohita*. The feed fingerlinggiven during the study was 5% / biomass weight / day with the feeding frequency of three times a day.

Digestive Enzymes Analysis: Analysis of the digestive enzymes activity using crude extract of the digestive tract of java barb referred to the Sandeepa and Ammani (2015), while the measurement of total protein activity referred to the method of Brandford (1976). Meanwhile protease activity and amylase activity referred to Anson's method (1938) and Rick and Stegbauer's methods (1984), respectively.

Parameters: The parameters observed included protein digestibility (ADCP) using the method of Fenucci (1981), efficiency of feed utilization (EFU), ratio of feed conversion (FCR) and ratio of protein efficiency (PER) using the Tacon's method (1995), relative growth rate (RGR) and survival rate (SR) using the NRC (1993) method, digestive enzyme activity, and water quality. The observed parameters were:

$$ADCP = 100 \left(\frac{\%\ Cr2O3\ \text{in diet}}{\%\ Cr2O3\ \text{in excreta}} \times \frac{\%\ \text{protein in excreta}}{\%\ \text{protein in diet}} \right)$$

$$EFU = \frac{\text{(Final body weight – Initial body weight)}}{\text{The amount of feed consumed}} \times 100\%$$

$$FCR = \frac{\text{The consumed feed weight}}{\text{(Final body weight + Total dead fish weight) – Initial body weight)}} \times 100\ \%$$

$$PER = \frac{\text{Final body weight – Initial body weight}}{} \times 100\%$$

The consumed feed weight x Protein content of feed

$$RGR = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight x Time experiment}} \times 100\%$$

$$SR = \frac{\text{Final body count}}{\text{Initial body count}} \times 100\%$$

Water quality: Observed parameters of water quality included temperature, pH and DO. They were enumerated daily using Water Quality Checker and ammonia. The measurement was conducted at the starting time, intermediate and at the end of the study using the APHA method (1995).

Statistical analysis: The observed parameter data (ADCP, EFU, FCR, PER, RGR, SR and digestive enzyme activity) were analyzed using ANOVA test. If the ANOVA analysis had significantly high ($P \leq 0.01$) or significantly ($P \leq 0.05$) influenced on those parameters, they were followed by Duncan's multiple region test to find out the mean difference among treatments (Steel *et al*., 1996). Parameters of water quality data were descriptively analyzed.

RESULTS AND DISCUSSION

The findings of digestive enzymes and the parameters of water quality were displayed in the Table 1, 2 and 3.

Table 1. Initial weight, final weight, ADCp, EFU, FCR, RGR, PER and SR of Java barb fingerling during the study.

Experiment Data	Treatments			
	A	B	C	D
Initial weight (g)	3.34±0.06	3.28±0.04	3.40 ±0.03	3.36 ±0.01
Final weight(g)	28.19±0,04^{c}	34.53±0,02^{b}	38.69±0,05^{b}	45.24±0,03^{a}
ADCp	52.63±0.03^{c}	62.14±0.07^{b}	75.28±0.06^{a}	63.89±0.07^{b}
EFU (%)	45.19±0.56^{c}	63.26±0.86^{b}	74.06±0.68^{a}	65.63±0.73^{b}
FCR	2.58±0.16^{c}	2.05±0.06^{b}	1.52±0.02^{a}	1.98±0.14^{b}
RGR (%)	1.87±0.24^{c}	2.36±0.21^{b}	3.78±0.14^{a}	2.64±0.74^{b}
PER	1.56±0.13^{c}	2.21±0.17^{b}	3.23±0.16^{a}	2.37±0.13^{b}
SR (%)	78.33±2.45^{c}	88.33±2.23^{b}	98.33±2.32^{a}	88.33±2.54^{b}

Note different superscripts of mean values show a significant difference ($P \leq 0.05$)

Table 2. Digestive enzymes values in the digestive system of Java barb fed with enrichment of *B. subtilis* probiotics during the study.

Activity (U g-1 protein)	Treatments			
	A	B	C	D
Total protein	2.26 ±0,13^{d}	3.23 ±0,16^{c}	5.28 ±0,18^{a}	4.18 ±0,25^{a}
Protease	1.65 ±0,15^{c}	1.83 ±0.21^{c}	4.32 ±0.27^{a}	2.75 ±0.26^{b}
Amylase	1.48±0,02^{c}	2.08±0,03^{b}	3.42±0.17^{a}	2.23 ±0.14^{b}

Note: different superscripts of mean values show a significant difference ($P \leq 0.05$)

Table 3. Water quality parameters during the study.

Treatment	Water Quality			
	Temperature (^{0}C)	pH	DO (mg/l)	NH_3 (%)
A	25 - 30	7.21 - 7.43	3.68 - 4.97	0.002 - 0.004
B	25 - 30	7.06 - 7.52	3.74 - 4.78	0.002 - 0.004
C	25 - 30	7.31 - 7.48	3.63 - 4.59	0.002 - 0.004
D	25 - 30	7.29 - 7.37	3.72 - 4.68	0.002 - 0.004
Feasibility	14-38*	6.50 - 8.5*	>2*	≤0.1*

Note : * Boyd (2003)

The results of observations of water quality parameters in Table 3 showed that the parameters of water quality during the study were still within the feasible range values according to the reference, it concluded that it was suitable for Java barb cultivation.

The results of the analysis of the variance of *B. subtilis* probiotic supplementation of the different doses had significantly ($P \leq 0.05$) influenced on digestibility of protein (ADCp) of Java barb fish fingerling (*Puntius javanicus*). Java barb fed with *B. subtilis* probiotic supplementation (0.75 - 2.25 x 10^{7} CFU g^{-1} feed) had higher ADCP values than without supplementation (0 CFU g^{-1} feed). It was suspected that *B. subtilis* has the ability to produce protease enzymes so that the digestive activity of the protein runs optimally. *B. subtilis* probiotics produce protease enzymes that can increase protein digestion activities (Verschuere *et al*., 2000; Ziaei-Nejad *et al*., 2006; Jafaryan *et al*., 2011). The highest ADCP value was obtained from Java barb fed C (1.50 x 10^7 CFU g^{-1} feed), this was thought to be an effective dose for *B. subtilis* to generate protease enzymes in the digestive tract of Java barb fingerling so that digestibility of feed protein runs maximum. This condition was supported by the results of the analysis of digestive enzymes on the observation of total proteins and proteases in the digestive tract of Java barb fingerlingfed C (Table 2) having the highest total protein and protease activity of (5.28 U g^{-1} protein and 4.32 U g^{-1} protein) compared to feed D (4.18 U g^{-1} protein and 2.75 U g^{-1} protein), B (3.23 U g^{-1} protein and 1.83 U g^{-1} protein) and A (2.26 U g^{-1} protein and 1.65 U g^{-1} protein).

Java barb (*P. javanicus*) fed with *B. subtilis* probiotic supplementation in the treatments B, C and D (0.75 - 2.25 x 10^7 CFU g^{-1} feed) had a higher EFU value (63.26 - 74.06%) compared to without supplementation in the treatment A (45.19%). This was supported by the results of research on observations of Java barb fingerling digestive enzymes (Table 2) which showed that feed with enrichment of *B. subtilis* probiotics can increase the activity of digestive enzymes of Java barb, therefore the efficiency of feed utilization can be maximized. The results of these studiesindicated that supplementation of *B. subtilis* probiotic in the feed can improve the efficiency of utilization of Java barb feed. This was confirmed by the opinion of El-Haroun *et al*. (2006) which stated that the addition of probiotics into the feed caused fish to be able to utilize nutrients more efficiently so as to improve the efficiency of feed utilization. Furthermore Lara-Flores *et al*. (2003) stated that the probiotics supplementation into the feeds could result in a higher efficiency of feed utilization compared to without the addition of probiotics. Moreover, Merrifield *et al.* (2010) suggested that the probiotics supplementation into the feed of tilapia and other species led to better nutrient absorption and efficiency of feed utilization. The findings of the same study were reported by Bogut *et al.*, (1998) on *Cyprinus carpio*, Wang, (2007) and Zhou *et al.* (2009) on *Litopenaeus vannamei* and Wu *et al*. (2012) on *Ctenopharyngodon idella*.

The FCR value of Java barb fed with *B. subtilis* probiotic supplementation (0.75-2.25 x 10^7 CFU g^{-1} feed) decreased further by 2.05 - 1.52 compared to no additions (0% / kg of feed) which was 2.58. The findings showed that *B. subtilis* supplementation in feed can reduce FCR due to protein digestibility and efficiency of feed utilization increases so that the feed conversion ratio decreases. Taoka *et al*. (2006), Bagheri *et al*. (2008), Wang *et al.* (2008[a]) reported that the *B. subtilis* probiotic supplementation in feed could reduce the feed conversion ratio of Salmon. Java barb fingerling fed as in the treatment C (1.50 x 10^7 CFU g^{-1} feed) resulted in the lowest FCR value indicated by the highest protein digestibility and efficiency of feed utilization (75.28% and 74.06%) compared to treatments D (63.89% and 65.63%), B (62.14% and 63.26%) and A (52.63% and 45.19%). The results of similar studies were reported on *Cyprinus carpio* (Wang and Xu, 2006), *Litopenaeus vannamei* (Wang, 2007; Zhou *et al*., 2009), *Ctenopharyngodon idella*, (Wu *et al*., 2012), *S. aurata* (Salinas *et al*. , 2005, 2006; Díaz-Rosales *et al*., 2006; Suzer *et al*., 2008; Avella *et al*., 2010).

The RGR value of Java barb fed with *B. subtilis* supplementation was 0.75 - 2.25 x 10^7 CFU g^{-1} feed increased from 2.38 - 3.78% / day compared to without supplementation (0 CFU g^1 feed) of 1.87% / day. The findings were the same as with the opinion of Merrifield *et al*. (2010) which stated that probiotic addition in feed increases fish growth effectively. Lara-Flores *et al*. (2003) reported that the probiotics supplementation into the feed produced higher growth compared to without the addition of probiotics. The results of the study by Taoka *et al*. (2006), Bagheri *et al.* (2008), Wang *et al*. (2008[a]) reported that the *B*. *subtilis* probiotic addition in the feed can increase the growth of Salmon. Java barb fed as in the treatment C (1.50 x 10^7 CFU g^{-1} feed) had the highest RGR value of 3.78% / day followed by the treatments D (2.64% / day), B (2.36% / day) and A (1.87% / day). The Java barb fed as in the treatment C had the highest RGR value, it was assumed that the dose of *B. subtilis* is 1.50 x 10^7 CFU g^{-1} feed was an effective dose of *B. subtilis* in increasing growth because at that dose the digestive enzymes in digestion of Java barb fingerling run optimally. The statement was supported by observations of digestive enzyme activity in Table 2 which showed that the highest digestive enzyme (total protein, protease and amylase) activity was obtained from Java barb fingerlingfed in the treatment C compared to other treatments. In addition, Java barb fed as in the treatment C had the highest ADCp and EFU values (75.28% and 74.06%) compared to treatments D (63.89% and 65.63%), B (62.14% and 63.26%) and A (52.63% and 45.19%). The results of a similar study were reported by Wang, (2007); Zhou *et al*. (2009); Wu *et al*. (2012); Macey and Coyne, (2005), Wang *et al*. (2005), Wang and Xu, (2006).

Java barb fingerling fed with *B. subtilis* supplementation of0.75 - 2.25 x 10^7 CFU g^{-1} feed had a higher PER value than without supplementation (0 CFU g^{-1} feed). The findings revealed that the *B. subtilis* addition in the feed could increase protein digestibility and the number of protease enzymes in the digestive tract of Java barb fingerling, so that the formation of proteins of the Java barb increased. Lara-Flores *et al*. (2003) suggested that the addition of probiotics into the feed can boost digestibility of protein and protein efficiency which causes an increase in fish protein. The same results were occured on sea bass (Tovar *et al*., 2010), *Cyprinus carpio* (Bogut *et al.,* 1998), *Litopenaeus vannamei* (Wang, 2007; Zhou *et al*. 2009) and *Ctenopharyngodon idella* (Wu *et al*., 2012).

The results of the variance analysis disclosed that the *B. subtilis* probiotic supplementation at different doses in the feed had significantly ($P \leq 0.05$) affected on the Java barb fingerling survival. The findings of this study indicated that the survival rate of Java barb rised with the supplementation of *B. subtilis* probiotic in the feed. It was suspected that *B. subtilis* probiotics can increase the body's resistance so as to improve the Java barb survival. Taoka *et al*. (2006), Bagheri *et al*. (2008), Wang *et al*. (2008[a]) reported that the *B. subtilis* probiotics addition in the feed can improve the survival of Salmon. The findings of a similar research were also reported by Wang, (2007), Avella *et al*. (2010), Wen-Ying *et al*. (2010) and Mohapatra *et al*. (2012).

The findings of the variance analysis revealed that the different dosages of *B. subtilis* probiotic supplementation in the feed had significantly ($P \leq 0.05$) influenced on the digestive enzymes activity of Java barb. Observations of digestive enzyme activities (Table 2) showed higher levels of digestive enzyme activities (total protein, protease, amylase) obtained on Java barb fed with *B. subtilis* probiotic supplementation (treatments B, C and D) compared to feed without supplementation (treatment A). The results of the same study was reported by Ziaei-Nejad *et al*. (2006). They suggested that the activity of digestive enzymes of shrimp (*Fenneropenaeus indicus*) fed with supplementation with *Bacillus* sp. was higher than those of without supplementation. The observation of the digestive enzyme activity of Java barb fingerling in the treatment C had a higher value of digestive enzyme activity than those of the treatments D, B and A. This was presumed to be a dose of probiotic *B. Subtilis* of 1.50 x 10^7 CFU g^{-1} feed was the best dose to boost the amount of microbes in the intestine digestive tract of Java barb fingerling. This finding was the same as with the study result of Rengpipat *et al*. (2000) which stated that the right dose of probiotics in the feed can increase the number of microbes in the intestine so that the enzymes activity in the digestive system increases.

Conclusion: Supplementation of *B. subtilis* into feed increased protein digestibility, growth and digestive enzymes activity of Java barb (*P. javanicus*) which was cultured in intensive aquaculture system. The dose of 1.50 x 10^7 CFU g^{-1} feed was the best dose of *B. subtilis* supplementation in the feed of Java barb (*P. javanicus*).

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