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Floating Net Aquaculture Engineering of Tiger Grouper (*Epinephelus fuscoguttatus*) on *Bacillus subtilis* Probiotic Supplementation in the Diet

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Abstract | Accumulation of feed waste from a long-term aquaculture practice has resulted in the deterioration of the aquaculture environment. Floating net aquaculture engineering of tiger grouper (*Epinephelus fuscoguttatus* Forsskål, 1775) on *Bacillus subtilis* supplementation in the diet is one of the solutions to overcome the deterioration of the aquaculture environment caused by the accumulation of dieting waste. The purpose of the research was to study the effects of *B. subtilis* supplementation in the diet on protein digestibility, the efficiency of diet utilization, growth, and activities of digesting enzymes of *E. fuscoguttatus*. The sampled fish has an average weight of 4.24 g ± 0.023 g per fish. Diet used in the study contained 45 % protein with the supplementation of various amounts of *B. subtilis* varying in (0, 2.5, 5, 7.5, 10, and 12.5) % per kg of diet as A, B, C, D, E, and F treatment, respectively. The results show that *B. subtilis* supplementation in the diet significantly affected on protein digestibility (ADC_P), the efficiency of diet utilization (EFU), feed conversion ratio (FCR), the protein efficiency ratio (PER), relative growth rate (RGR), survival rate (SR) and activities of digesting enzymes of *E. fuscoguttatus*. In conclusion, the optimum amounts of *B. subtilis* in the diet on ADC_P, EFU, FCR, PER, RGR, and SR were at (7.34, 7.36, 7.18, 7.5, 7.48, and 7.5) % per kg of diet, respectively.

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Introduction

Karimunjawa Islands are located in Jepara District, Province of Central Java (Indonesia), located approximately 45 miles from Jepara City. Karimunjawa Islands consists of 27 islands. They sit on 5°40' to 5°57' LS and 110°4' to 110°40' BT, and have an area of 107.225 ha, consisting of 100.105 ha of the sea and 7.120 ha of land. Only 10 % of the total sea (100.105 ha) is used as tiger grouper (*Epinephelus fuscoguttatus* Forsskål, 1775) floating net aquaculture (Samidjan and Rachmawati, 2018). This aquaculture has been deteriorating since there occurs dieting waste accumulation, resulted from long-term practice of aquaculture. One of the solutions to solve the problems is by implementing floating net aquaculture engineering and by dieting *E. fuscoguttatus* with *Basillus subtilis* probiotic to increase the efficiency of diet utilization. In turn, it can reduce dieting waste and increase fish production.

According to Iribarren *et al*. (2012), probiotics in the

diet can increase the growth and efficiency of diet utilization; therefore, it can reduce dieting waste in the environment. Some probiotic bacteria applied in the aquaculture whether fresh, brackish, or saline water aquaculture are *Bacillus* sp. (Boonthai *et al.*, 2011), *Bacillus subtilis* (Keysami *et al.*, 2012; Merrifield *et al.*, 2010; Mohapatra *et al.*, 2012), *Enterococcus faecium* (Gopalakannan *et al.*, 2011) and *Lactobacillus acidophilus* (Wang, 2011).

Probiotics can improve the digestion of fish by producing digesting enzymes in the intestine to increase growth (Gatesoupe, 2008; Rachmawati *et al.*, 2018). One of the bacteria which can improve the digestion of fish is *Bacillus* sp. Furthermore, *Bacillus* sp. can excrete protease, lipase, and amylase enzymes (Wang and Xu, 2006). Some studies of *Bacillus* sp. probiotic supplementation in the diet reveal the impact on growth, efficiency of diet, nutrient digestion, the effectivity of digesting enzymes, improve beneficial organism, inhibiting of a pathogen, and increasing of the immune system. The impact was observed on such fish species as *Oncorhynchus mykiss* Walbaum, 1792 (Merrifield *et al.*, 2010), *Siganus rivulatus* Forsskål and Niebuhr, 1775 (El-Dakar *et al.*, 2007), *Ctenopharygodon idella* Valenciennes in Cuvier and Valenciennes, 1844 (Wang, 2011), *Cyprinus carpio* Linnaeus, 1758 (Gopalakannan *et al.*, 2011), *Penaeus monodon* Fabricius, 1798 (Boonthai *et al.*, 2011), *Macrobrachium rosenbergii* De Man, 1879 (Keysami *et al.*, 2012), *Labeo rohita* F. Hamilton, 1822 (Mohapatra *et al.*, 2012).

In aquaculture, probiotics can be added to the diet as a supplement and additive (Avella *et al.*, 2010; Suzer *et al.*, 2008; Ziaei-Nejad *et al.*, 2006). The study of *B. subtilis* probiotic supplementation in the diet for *E. fuscoguttatus* aquaculture is still very limited; therefore, additional studies on this topic are still needed. The objectives of the study were to identify the effects of *B. subtilis* probiotic supplementation in the diet on protein digestibility, the efficiency of diet utilization, growth, and activity of digesting enzyme of *E. fuscoguttatus* raised in the floating net aquaculture system.

Materials and Methods

Preparation of floating net aquaculture

E. fuscoguttatus was reared in a Floating Net Aquaculture System in the Karimunjawa Islands with the dimension of 4 m × 4.5 m × 4 m. The net was mostly submerged, 4 m × 3 m x 3 m. The net was tied onto the raft with a size of 9 m × 9 m. The raft was placed in a depth of 18m. The raft was made of 24 logs, each with a dimension of 10 cm ×14 cm × 400 cm. The raft was assembled with bolts with a size of 18 cm. The floating used styrofoam as many as 16 pieces. The floating net was made of polyethylene with a mesh size of ¾ inch.

Preparation of the fish samples

E. fuscoguttatus used in the study had an average weight of 4.24 g ± 0.023 g per fish. The fish was collected from the Center for Brackish Water Aquaculture, Jepara, Central Java, Indonesia, and selected to get healthy fish, without any deformation, of a uniform size and weight (Rachmawati *et al.*, 2017). The observation was done weekly by sampling as many as 20 fish in each treatment.

Bacillus subtilis

B. subtilis specimen was obtained by isolating the bacteria from *E. fuscoguttatus* raised in the Center for Brackish Water Aquaculture, Jepara, Central Java, Indonesia. The preparation of bacteria specimen followed the method of Sandeepa and Ammani (2015). Firstly, the sterilized bacteria were planted in deMann Rogosa Sharpe Agar (MRS). Secondly, the bacteria cells were mixed with commercial gel to get the dose of *B. subtilis* for the treatments (A: 0 % per kg diet, B: 5 % per kg diet, C: 10 % per kg diet, and D: 15 % per kg diet).

Feed preparation

Diet used in the study was a manufactured diet containing 45 % protein as a basic diet. Addition of the probiotic *B. subtilis* to the basic diet was performed by boiling cassava flour (*Manihot esculenta* Crantz.) with water until it became a paste; after boiling the paste was cooled off. Then the paste was mixed with the probiotic *B. subtilis* with the amounts adjusted to the different treatment doses (A: 0 % / kg feed, B: 2.5 % / kg of feed, C: 5 % / kg of feed, D: 7.5 % / kg of feed, E: 10 % / kg of feed and F: 12.5 % / kg of feed). Then the basic diet was coated with the paste, and dried in the open air, and stored in a freezer at -20 °C (Adineh *et al.*, 2013). The *E. fuscoguttatus* was raised for 60 d and fed four times a day with the amounts of diet as much as 4 % biomass weight per day.

Digesting enzyme analysis

Raw extract of *E. fuscoguttatus* digesting system was

used to measure digesting enzyme activities in various treatments. The whole digesting system was collected from fish and homogenized with de-ionized water (1:10). Then it was centrifuged at 5 000 g for 20 min and left at the temperature of 4 °C. The supernatant was carefully separated and filtered with 0.45 mm mesh (Sartorius, Jerman). The analyses of various enzymes were based on the method described in the literature (Sandeepa and Ammani, 2015). The measurement of total protein activity, protease activity, and amylase activity were based on the methods of Bradford (1976) and Rick *et al.* (1984).

Observed parameters

Parameters that were observed included protein digestibility (ADC_P), efficiency of diet utilization (EFU), diet conversion ratio (FCR), and protein efficiency ratio (PER), raw growth relative (RGR), survival rate (SR). Fenucci (1981) method was used to analyze (ADC_P), while Tacon *et al.* (2002) method was used to analyze EFU method. The observed with Equation 1 to Equation 6 as follow:

$$ADC_P = 100 \times [(\%Cr_2O_3 feed \times \% protein feces)/ (\% Cr_{2O3} feces \times \% protein feed)] \quad ....(1)$$

$$EFU = \{(Final\ weight - Initial\ weight) / the\ amounts\ of\ feed\ consumed\} \times 100\ \% \quad ...(2)$$

$$FCR = \{the\ amounts\ of\ feed\ consumed / [(Final\ weight + Total\ weight\ fish\ death) - Initial\ weight]\} \quad ....(3)$$

$$PER = \{(Final\ weight - Initial\ weight)/ (the\ amount\ of\ feed\ consumed\ x\ Protein\ content\ of\ feed)\} \times 100\ \% \quad ....(4)$$

$$RGR = \{(Final\ weight - Initial\ weight) / (initial\ weight\ x\ time\ experiment)\} \times 100\ \% \quad ...(5)$$

$$SR = (Final\ count / Initial\ count) \times 100\ \% \quad ... (6)$$

Water quality observation

Water quality parameters that were observed consisted of temperature, pH, dissolved oxygen (DO), ammonia, and salinity. The method used to analyze those parameters was performed according to APHA (2005). The measurement of salinity was conducted every day.

Statistical analysis

ANOVA was used to analyze ADC_P, EFU, FCR, PER, RGR, SR, activity of digesting enzyme, and blood profile. Duncan double analysis was also used to determine the significance of the test ($P < 0.05$) (Steel *et al.*, 1996). The calculation used SPSS version 21 statistical software (SPSS Inc, Chicago, IL, USA). The polynomial orthogonal test was used to determine the optimum dose of *B. subtilis* in the diet. Water quality was descriptively analyzed.

Results and Discussion

The results were shown in the following Tables 1 and 2.

Table 1: *Data of initial weight, final weight, ADC_P, EFU, RGR, PER, and SR of Tiger Grouper (Ephinephelus fuscoguttatus).*

Experimental data	Treatments			
	A	B	C	D
Initial weight (g)	4.24 ±0.02	4.14 ±0.02	4.28 ±0.03	4.30 ±0.01
Final weight(g)	22.26±0.03^c	24.78±0.02^b	28.89±0.04^a	25.54±0.02^b
ADC_P	50.79±0.08^c	60.54±0.09^b	75.89±0.05^a	62.32±0.06^b
EFU (%)	55.32±0.54^c	65.26±0.97^b	72.26±0.89^a	66.75±0.83^b
FCR	2.54±0.03^c	2.18±0.06^b	1.73±0.07^a	2.03±0.07^b
RGR (%)	2.18±0.20^c	2.59±0.45^b	3.54±0.37^a	2.89±0.26^b
PER	1.35±0.04^c	1.58±0.06^b	2.00±0.05^a	1.73±0.07^b
SR (%)	76.67±2.39^c	88.33±2.13^b	93.33±2.24^a	86.33±2.89^b

A: suplementation of B. subtilis probiotic with the dosage of 0 % per kg diet; B: suplementation of B. subtilis probiotic with the dosage of 2.5 % per kg diet; C: suplementation of B. subtilis probiotic with the dosage of 5 % per kg diet; D: suplementation of B. subtilis probiotic with the dosage of 7.5 % per kg diet; E: suplementation of B. subtilis probiotic with the dosage of 10 % per kg diet; F: suplementation of B. subtilis probiotic with the dosage of 12.5 % per kg diet.

The value of ADC_P with the supplementation of *B. subtilis* probiotic in the diet (2.5 % to 12.5 % per kg diet) was higher than that without supplementation (0 % per kg diet). The highest value of ADC_P was observed at treatment D with the supplementation of 7.5 % per kg diet; the value was 78.32 %. It was followed by treatments, C (67.89 %), E (64.21 %), F (63.17 %), B (62.54 %) and A (50.79 %), respectively. The higher value of ADC_P in the treatment D (7.5 per kg diet) was due to the right dose of *B. subtilis* probiotic in the diet to produce protease enzyme in the intestine; in turn, it made optimum protein digestibility, as shown in Table 2. The activity of digesting enzyme in treatment D (7.5 % per kg diet) was the highest compared to treatments C, E, F, B, and A. Wang *et al.* (2008) reported that *Bacillus* sp. can excrete protease enzyme. Moreover, El–Haroun *et al.* (2006), Jafaryan *et al.* (2011), and Verschuere *et al.* (2000) suggested that *B. subtilis* can produce a protease enzyme that increased the activity of protein

digestibility. The polynomial orthogonal test resulted in the optimum dose of *B. subtilis* at 7.34 % per kg diet with the value of ADCp as much as 72.35 % (Figure 1).

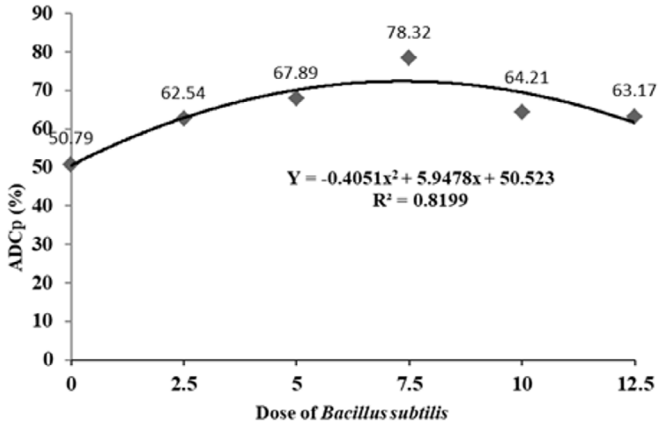

Figure 1: *Graph of the polynomial orthogonal test for ADC_p (%) of E. fuscoguttatus.*

Table 1 shows that *E. fuscoguttatus* fed with the dose of *B. subtilis* probiotic in the diet of 2.5 % to 12.5 % per kg diet has the value of EFU as much as 63.26 % to 80.75 % higher than that of without the supplementation (0 % per kg diet) with the value 55.32 %. As in the finding of the Lara-Flores *et al.* (2003) study, the supplementation of *B. subtilis* probiotic in the diet can increase nutrient efficiency. The finding was also supported by the observation of digesting enzymes of *E. fuscoguttatus.* As shown in Table 2 that the supplementation of *B. subtilis* probiotic in the diet can increase the activity of the enzyme, in turn, it can increase the efficiency of diet utilization. Moreover, Bogut *et al.* (1998) also reported that the supplementation of *B. subtilis* probiotic in the diet resulted in higher than without the supplementation. Merrifield *et al.* (2010) also found the same effect in the study of tilapia and other species. Similar results were also reported in the study of *C. carpio* (Bogut *et al.*, 1998), *Litopenaeus vannamei* Boone, 1931 (Zhou *et al.*, 2009) and *Ctenopharyngodon idella* Valenciennes, 1844 (Wu *et al.*, 2012). The polynomial orthogonal test shows that the optimum dose of *B. subtilis* probiotic was 7.36 % per kg diet with the value of 75.39 % efficiency of diet utilization (Figure 2).

E. fuscoguttatus fed with the supplementation of *B. subtilis* probiotics in the diet of 2.5 % to 12.5 % per kg diet has a higher value of RGR than that without the supplementation (0 % per kg diet). The supplementation of *B. subtilis* in the aquaculture system can improve growth (Mohapatra *et al.*, 2013). The highest value of RGR was obtained from the treatment D (7.5 % per kg diet) with the value of 4.89 % per day, followed by treatment C (5 % per kg diet) with the value of 3.84 % per day, E (10 % per kg diet) with the value of 3.46 % per day, F (12.5 per kg diet) with the value of 2.89 % per day, B (2.5 % per kg diet) with the value of to 2.59 % per day and A (0 % per kg diet) with the value of 2.18 % per day. The highest value of RGR in the treatment D (7.5 % per kg diet) was thought the effective dose of *B. subtilis* to increase the activity of digesting enzyme in the intestine; therefore, it can increase growth. The findings were supported by the data of digesting enzyme, as shown in Table 2. The results show that the highest activity of digesting enzyme was obtained in the treatment D (7.5 % per kg diet), followed by C (5 % per kg diet), E (10 % per kg diet), F (12.5 per kg diet), B (2.5 % per kg diet) and A (0 % per kg diet. Similar results were reported by Wang (2011) and Zhou *et al.* (2009) in the study of *L. vannamei;* and Wu *et al.* (2012) in the study of *C. idella.*

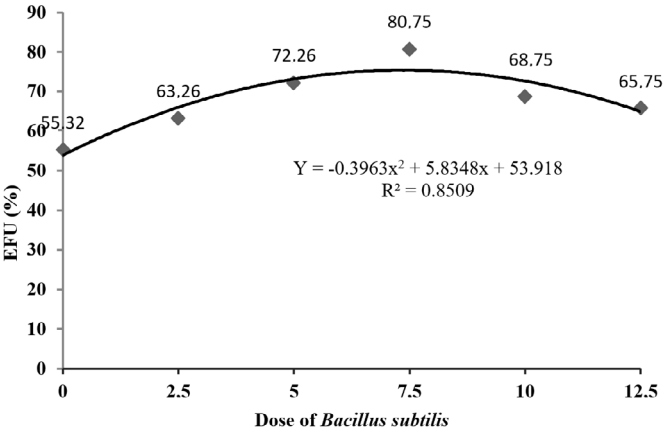

Figure 2: *Graph of the polynomial orthogonal test for EFU (%) of E. fuscoguttatus.*

The supplementation of *B. subtilis* probiotics in the diet can bring the fish to grow faster than without the supplementation (Lara-Flores *et al.*, 2003). Some studies also have found that the supplementation of *B. subtilis* probiotic in the aquaculture system can increase growth, as reported by Macey and Coyne (2005) and Wang and Xu (2006). The supplementation of *B. subtilis* probiotic in the aquaculture system can also increase the performance of the fish, growth, immunostimulation, and disease resistance (Merrifield *et al.*, 2010), as well as prolong the survival rate of the larvae after hatching (Ringø and Gatesoupe, 1998). The result of the polynomial orthogonal test resulted in the optimum dose of *B. subtilis* on RGR of 7.18 % per kg diet with the value of RGR as much as 4.16 % (Figure 3).

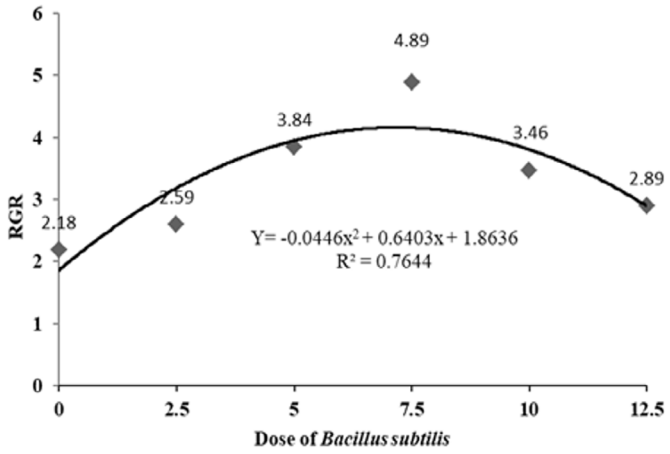

Figure 3: *Graph of the polynomial orthogonal test for RGR (% per day) of E. fuscoguttatus.*

Protein efficiency ratio of *E. fuscoguttatus* diet with the supplementation of *B. subtilis* probiotics in the diet of the dose 2.5 % to 12.5 % per kg diet ranged from 2.58 to 3.73 that were higher than without of the supplementation (1.85). The highest PER value was obtained by *E. fuscoguttatus* in treatment D (7.5 % per kg of feed). It was presumed that because *E. fuscoguttatus* in treatment D (7.5 % per kg diet) had the highest ADCp value compared to other feed treatments as seen in the observations of this study (Table 1). The supplementation of *B. subtilis* probiotics in the diet can increase protein digestibility and protein efficiency that can explain the improvement of diet efficiency (Lara-Flores *et al.*, 2003). A similar observation was done in the *C. idella* (Wu *et al.*, 2012). The highest protein efficiency ratio was 3.38 obtained from the *B. subtilis* probiotic dose of 7.48 % per kg diet (Figure 4).

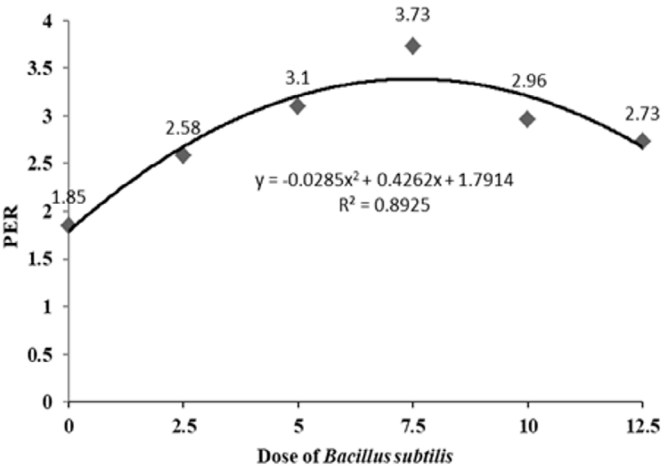

Figure 4: *Graph of polynomial orthogonal PER of E. fuscoguttatus*

Table 1 shows that *E. fuscoguttatus* fed with the supplementation of *B. subtilis* probiotic in the diet at 2.5 % to 12.5 % per kg diet has low FCR (1.32 to 2.18) compared to FCR without the supplementation (2.54). It means that the study of the supplementation of *B. subtilis* probiotics in the diet can increase FCR. The lowest FCR was obtained in the treatment D (7.5 % per kg diet), this is thought to have the highest values of ADCp and EFU at 78.32 and 80.75, respectively, compared to the treatment C (67.89 ADCp and 72.26 EFU), E (64.21 ADCp and 68.75 EFU), F (63.17 ADCp and 65.75 EFU), B (62.54 ADCp and 63.26 EFU) and A (50.79 ADCp and 55.32 EFU). Similar results were obtained from some studies in the species of *S. aurata* (Díaz–Rosales *et al.*, 2006; Salinas *et al.*, 2006; Suzer *et al.*, 2008), *C. carpio* (Wang and Xu, 2006), *C. idella* (Wu *et al.*, 2012). The highest FCR was 1.32 and obtained at the *B. subtilis* dose of 7.5 % per kg diet (Figure 5).

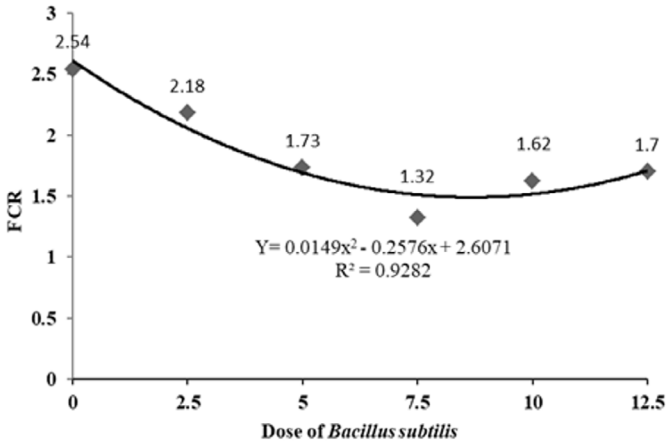

Figure 5: *Graph of polynomial orthogonal FCR of E. fuscoguttatus.*

The results of ANOVA showed that the supplementation of *B. subtilis* probiotic in the diet has a significant effect ($P < 0.05$) on the survival rate of *E. fuscoguttatus*r. It can increase the survival rate. It was suggested that the supplementation of *B. subtilis* probiotic in the di*et al*so increase the immune system of the fish. The finding was supported by (Shapawi, 2007) that the supplementation of *B. subtilis* probiotic in the aquaculture system. Some studies that resulted in similar results were conducted by Avella *et al.* (2010), Iribarren *et al.* (2012), Wen-Ying *et al.* (2010). Similar data were also reported for the species of *L. rohita* (Ghosh *et al.*, 2002), *Sciaenops ocellatus* Linnaeus, 1766 (Li *et al.*, 2006), *Paralichthys olivaceus* Temminck and Schlegel, 1846 (Taoka *et al.*, 2006), *C. carpio* (Wang and Xu, 2006), and *Fenneropenaeus indicus* H. Milne-Edwards, 1837 (Ziaei-Nejad *et al.*, 2006). The result of the polynomial orthogonal test shows that the optimum dose of *Bacillus subtilis* on survival rate was at the dose of 7.5 % per kg diet with

the level of SR of 93.33 % (Figure 6).

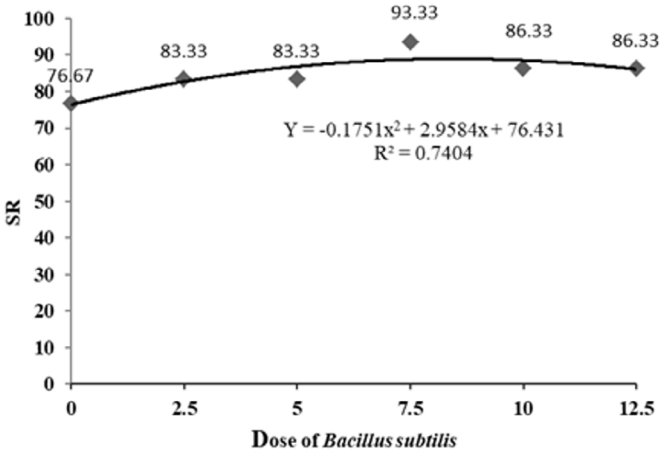

Figure 6: *Graph of polynomial orthogonal SR (%) of E. fuscoguttatus.*

Table 2 shows that the supplementation of *B. subtilis* probiotic in the diet has a significant effect ($P < 0.05$) on the enzyme activity of *E. fuscoguttatus*. The enzyme activity was higher with the supplementation of *B. subtilis* probiotic in the diet treatments B, C, D, E and F compared to the enzyme activity without the supplementation as in the treatment A. The highest enzyme activity in the digesting system was in treatment D (7.5 % per kg diet). It was suggested that the dose at the level of 10 % per kg diet was the optimum dose of *B. subtilis* in the diet to produce digesting enzyme. A similar result has been reported by Ziaei-Nejad *et al.* (2006) that the supplementation of *B. subtilis* probiotic in the diet for shrimp (*F. indicus*) was higher than without the supplementation.

Table 2: *Data of digesting enzyme in the digesting system of Tiger Grouper with the supplementation of B. subtilis probiotic in the diet.*

Activity (U g^{-1} protein)	Treatments			
	A	B	C	D
Total protein	2.43 ±0.02^{d}	4.05 ±0.02^{c}	5.28 ±0.03^{a}	3.23 ±0.15^{b}
Protease	1.34 ±0.09^{d}	1.75 ±0.03^{c}	2.68 ±0.03^{a}	2.13 ±0.13^{b}
Amylase	1.48±0.02^{d}	1.98±0.03^{c}	2.98 ±0.04^{a}	2.39±0.02^{b}

Notes: *A: suplementation of B. subtilis probiotic with the dosage of 0 % per kg diet; B: suplementation of B. subtilis probiotic with the dosage of 2.5 % per kg diet; C: suplementation of B. subtilis probiotic with the dosage of 5 % per kg diet; D: suplementation of B. subtilis probiotic with the dosage of 7.5 % per kg diet; E: suplementation of B. subtilis probiotic with the dosage of 10 % per kg diet; F: suplementation of B. subtilis probiotic with the dosage of 12.5 % per kg diet.*

Parameters of water quality during the study of *E. fuscoguttatus* in the floating aquaculture system were in viable condition (Table 3).

Table 3: *Water quality of parameter E. fuscoguttatus in the floating aquaculture system.*

Parameter	Unit	Range	Reference
Temperature	° C	27.5 to 30.5	26.0 to 33.0[a]
pH		7.2 to 8.1	7.0 to 8.2[a]
Salinity	ng L^{-1}	25.0 to 30.5	25.0 to 32.0[a]
Dissolved oxygen	mg L^{-1}	5.27 to 6.62	3.0 to 7.0[a]
NH_3	mg L^{-1}	0.011 to 0.017	0.02
NO_2	mg L^{-1}	to 0.05	0.1

Note : [a] Shapawi *et al.* (2007)

Conclusions and Recommendations

The supplementation of *B. subtilis* probiotic in the diet has a significant effect on protein digestibility, efficiency of diet utilization, growth, survival rate, and enzyme activities of *E. fuscoguttatus*raised in the floating aquaculture system. The optimum amounts of *B. subtilis* in the diet on $ADC_{P,}$ EFU, FCR, PER, RGR, and SR were at (7.34, 7.36, 7.18, 7.5, 7.48, and 7.5) % per kg of diet, respectively.

Acknowledgements

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Novelty Statement

Some previous studies of *Bacillus* sp. probiotic supplementation in the diet on fish growth, showed an efficiency of diet, nutrient digestion, digesting enzymes' effectivity, improved beneficial organism, inhibiting the pathogen, and increasing of the fish immune system. Nevertheless, the research on *Bacillus subtilis* probiotic supplementation in the diet of Tiger Grouper (*Epinephelus fuscoguttatus* Forsskål, 1775) has never been carried out. The conclusion of this study shows that *B. subtilis* supplementation in the diet of Tiger Grouper (*E. fuscoguttatus*) gives beneficial effect on increased protein digestibility, the efficiency of diet utilization, relative growth rate, survival rate, and activities of digesting enzymes.

Author's Contribution

DR conceptualized and designed the study, elaborated the intellectual content, performed literature search, data acquisition, data analysis, statistical analysis, and manuscript preparation. JH defined the intellectual content, literature search, and manuscript review. OA and RHS defined the intellectual content, literature search, manuscript review, and guarantor. TE performed, literature search, manuscript review, and manuscript editing. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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