Effect of Probiotic Bacillus subtilis Endospore on The Immune System of Leukocytes Respiratory Burst Activity (RBA) in Grouper (Epinephelus coioides)

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ABSTRACT

Probiotics are live microbes that can help protect and maintain the health of the host by modifying the microbial community or associating with the host, increasing the response to disease, improving nutrition, and utilizing feed. Probiotics have properties to increase feed efficiency and increase non-specific immunity in fish. Probiotic administration allows fish to achieve optimal growth and increase immunity to disease. The purpose of this study was to determine the effect of probiotic *Bacillus subtilis* endospore administration on the leukocytes respiratory burst activity (RBA) immune system in grouper (*Epinephelus coioides*). The results of the research showed that the administration of probiotics in the grouper (*Epinehelus coioides*) feed with the probiotic dose of *Bacillus subtilis* in treatment B (0.1% *Bacillus subtilis*) and treatment C (1 % *Bacillus subtilis*) had a significantly effect on Leukocytes Respiratory Burst Activity (RBA) compared to treatment A (0% *Bacillus subtilis*). The RBA values in treatment B (1.91) and treatment C (1.98) were significantly different start from 10 days of rearing time. While the best dose for the RBA value is treatment C (5.03) with an elapsed time 30 days.

Keywords : Grouper, Feed, Probiotic, *Bacillus subtilis*, Immune System, Leukocytes, RBA

Introduction

Animal growth especially in fish, depends on the digestion and absorption of food nutrients. The intestine plays an important role in nutrient absorption, protection, and it responsible for producing active endogenous molecules. The intestinal wall found in fish has four layers, namely lumen, submucosa, muscle, and serosa (Cerezuela et al., 2013).

Disease control caused by bacterial infection in fish is usually done by giving antibiotics. Continuous administration of antibiotics can result in bacterial resistance to these types of antibiotics. Efforts to control disease using antibiotics have been banned. The use of antibiotics in addition to causing bacterial resistance to these types of antibiotics, can also cause residual antibiotics in commercial fish that are not detected so that they can cause allergies and poisoning that can harm consumers (Cabello, 2006).

Probiotics are live microbes that can help protect and maintain the health of the host by modifying the microbial community or associating with the host, increasing the response to disease, improving nutrition, and utilizing feed Probiotics have properties to increase feed efficiency and increase non-specific immunity in fish. The administration of this probiotic allows fish to achieve optimal growth (Talpur et al., 2014) and increase immunity to disease (Muñoz-Atienza et al., 2015).

Implementation of probiotics to feed is one way to increase the body's resistance of fish (Geng et al., 2011). Several research results show that the use of *Bacillus sp*. as a probiotic has been able to increase the growth of fish (Ferdynan Sumule & Trisnawati Tobigo, 2017), immune response traits (Zaineldin et al., 2018) and reduce water pollution (Olmos & Paniagua-Michel, 2014).

Leukocytes are important part of the immune system preventing of the invation by different pathogens (Naidenko et al., 2020). Analysis of leukocytes is one of the easiest immune parameters to measure although is only one of small block of immunity. The leukocyte respiratory burst activity is an indicator of innate immunity in mammals (Rossi et al., 1982). According to scientists leukocyte respiratory burst activity associated with increased oxygen consumption. Right now, a breath explosion also related to cytokine release and inflammatory response in fish (Abreu et al., 2009). Leukocytes respiratory burst activity is essential in fish defence that considered potent bactericides which actively destruct invasive pathogen (Biller & Takahashi, 2018). Research on the value of leukocytes proliferation in fish species can provide an overview of health status and process on occurence of disease (Afiyanti et al., 2019). Increased value of leukocyte proliferation indicates a response of body resistance to disease causing antigen (Diepen, 1993). In addition, the increase in leukocyte proliferation also indicates the occurrence of stress (Maftuch, 2018). The purpose of this study was to determine the effect of probiotic *Bacillus subtilis* endospore administration on the immune system respiratory burst activity (RBA) in grouper (*Epinephelus coioides*).

Material and Method

1. Feed Preparation Treatment

Fish feed consists of fish meal, squid meal, fish oil, , glutinous rice flour, soy protein, skinless soybean meal, and vitamins and minerals premix. The three feeds have different probiotic composition, treated by coating the feed with egg white. Probiotics *Bacillus subtilis* in the form of endospores were obtained from Laboratory of Prof. Hsieh, NPUST, Pingtung,

Taiwan. For feed A (control) with (0 % probiotic *Bacillus subtilis*) the feed was only coated with egg white without the addition of probiotic *Bacillus subtilis*. While for treatment B feed (0.1 % of probiotic *Bacillus subtilis*) and treatment C feed (1 % of probiotic *Bacillus subtilis*) the treatment feed was coated with egg white and probiotics according to the dose per kg of feed. Each treatment feed was examined for the concentration of *Bacillus subtilis* every week to ensure the concentration of the feed dose did not change. Total Viable Count (Log TVC) in treatment A, B and C were maintained at Log TVC 0; 2.5 X 10^5; and 7.9 X 10^6.

2. Calculating Total Bacteria

Total viable count (TVC) in sea water tank estimated total number of bacteria in sea water rearing tank. Total Viable count (TVC) in the seawater tank counting using protocol (Araujo et al., 2015). Briefly, samples from seawater tanks should be incubated at 37˚C for 24 hours. Each treatment was repeated 3 times. The total number of colonies was calculated as Colony Forming Units (CFU) which calculated using the formula: CFU = Number of colonies x Dilution factor /sample volume (ml) (Hameed et al., 2015).

3. Mixing Feed with Probiotics

The probiotics used are probiotics in the form of *Bacillus subtilis* endospores. The feed administration is artificial feed with a protein content of ± 38 %. Egg whites were still given to the control treatment (A) without probiotics. Probiotics were mixed into the artificial feed as much as 0.1% (B) and 1% (C). Before being mixed into feed, probiotics are attached in egg whites as a binder. After that the probiotics are mixed into the feed and stirred until evenly distributed. Then the feed was dried using a low temperature oven (40^0 C) until the feed was dry. Then each feed is packaged in a tightly sealed plastic bag that has been labeled. Furthermore, the packaged feed is stored in a refrigerator at a temperature of -5 ^{0}C.

4. Treatment Design

This research was conducted at the Fish Nutrition Laboratory, National Sun Yat-sen University (NSYSU), ROC, Taiwan. The fish used in this study was the grouper (Epinephelus coioides). The grouper was first kept in a tank for 10 days for the adaptation process. The container used in this study was a measuring tank 100 x 60 x 50 cm with 3 treatments A (0 % probiotic *Bacillus subtilis*), B (0.1% probiotic *Bacillus subtilis*) and C (1 % probiotic *Bacillus subtilis*) per kg of feed. The test fish had weight about 25 gr and the treatment was carried out

with three replications. During the rearing of the grouper using a closed aeration system in an indoor tank. Fish farming was carried out for 30 days with the frequency of feeding three times a day. Water temperature during rearing condition maintained at a 25–30 °C. Stocking test fish in each tank was 20 fish/tank. Furthermore, for fish immune system assay were taken randomly as many as 2 tails for each treatment then adapted to the tank and fasted for one day.

5. Immune System Sampling Preparation

Fish samples preparation were treated using the procedure ((Hu et al., 2015). Briefly, the first stage anesthetizes the fish using a benzocaine solution with a concentration of 0.4%. The skin and gills are sterilized first. As for the stomach is slashed and the contents removed. Head kidney cells sample collection were obtained by following protocol of (Secombes, 2011). In summary, aseptic head kidney was placed in a petri dish containing L-15 media. The head kidney was crushed and filtered using a nylon sieve with a mess size of 100 μm. The resulting cell suspension will cover the Percoll gradient of 34%/51% v/v. then centrifuged at 400 rpm for 30 minutes at 40 ^{0}C. The middle layer was taken and rinsed twice using L-15 media. Then the suspension results are calculated with a concentration of 2 x 10^5 cells per ml.

The blood sample for respiratory burst activity (RBA) following the protocol (Secombes¸ 2011) with some modifications. Briefly, blood samples (1 x 10^5 cells) were incubated with 100 μl NBT in culture medium for 30 minutes at 28 ^{0}C. Then the supernatant was removed and fixed with 99.9% methanol for 10 minutes. Each well was rinsed twice with 70% (v/v) methanol. The cells were allowed to dry in the air. The reduced NBT in the form of formazan was dissolved using a solution of potassium hydroxide and dimethyl sulfide. Then measured using a spectrophotometer at a wavelength of 620 nm.

Leukocyte proliferation was determined by the protocol (Daly et al., 1995). In Summary, cell suspensions with a concentration of 2 x 10^5 cells/ml were cultured for 48 hours with LPS. Then 20 μl MTT (5 mg/ml PBS) was added to each well and incubated for 4 hours at 27 ^{0}C. The tissue culture plates were centrifuged at 500 rpm for 10 min and then the liquid supernatant was carefully removed without disturbing the pellet or formazan precipitate. The pellet were dissolved with each addition of 200 μl of DMSO (Sigma). The contents of the well are then mixed using a pipette. Ten minutes later, the formazan was read by using a Titer Multiscan (Flow) plate reader at a wavelength 595 nm.

Results and Discussion

1. Results

a. Total Bacteria in Rearing Media

Total bacteria in the grouper rearing media depicted by log TVC. The results of of total bacteria in the rearing media can be explained that addition of probiotics *Bacillus subtilis* causes changes in the Log TVC of seawater tank. Log TVC increased with increasing culture period. The log TVC in treatment B and C were higher than treatment A. Log TVC in treatment A, B and C after 10 days of rearing condition was log 0.24 x 10^3; log 0.47 x 10^3; and log 1.74 x 10^3 respectively. Log TVC in treatment A, B and C after 20 days of rearing condition were log 0.26 x 10^3; log 1.45 x 10^3 and log 1.89 x 10^3. Then for 30 days of rearing condition were log 0.67 x 10^3; log 3.45 x 10^3 and log 4.51x 10^3 respectively for treatment A, B, and C.The population level of living bacteria is somewhat higher in the treatment C on all accession time, then followed by treatment B (Figure 1).

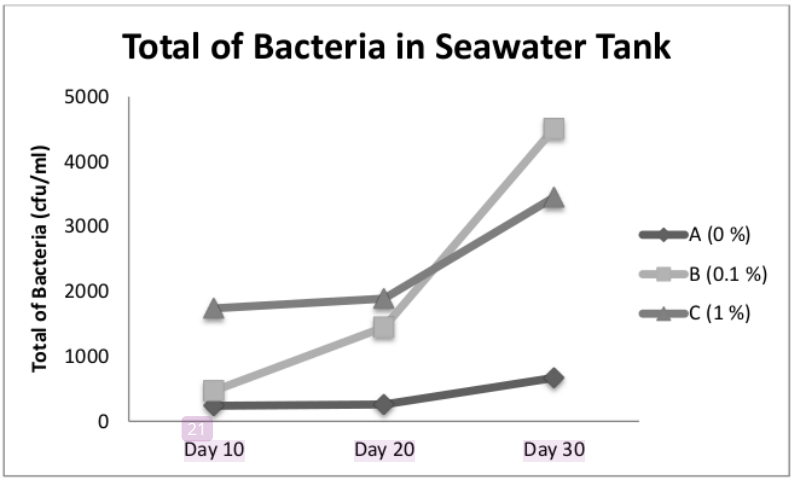

Figure 1. Total Bacteria in Rearing Media

b. Body Weight of The Grouper (*Epinephelus coioides*)

Administration of probiotic *Bacillus subtilis* in treatment B and C did not significantly affect the growth of grouper (Epinephelus coioides) when reared for 30 days, even though in a graphical trend (Figure 2) body weight of the grouper (*Epinephelus coioides*) in the treatment C (1 % Probiotic *Bacilllus subtilis*) and B (0.1% probiotic *Bacilllus subtilis*) were higher than treatment A (Control).

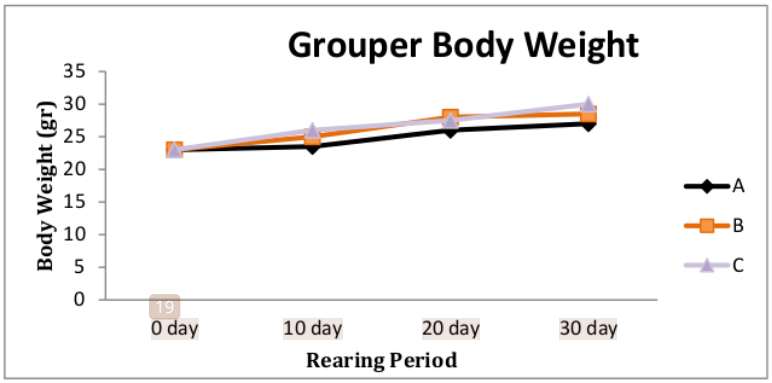

Figure 2. Grouper (*Epinephelus coioides*) Body Weight

c. Leukocytes Respiratory Burst Activity (RBA) of Grouper (*Epinephelus coioides*)

Feeding probiotic *Bacilius subtilis* had a significant effect on Leukocytes Respiratory Burst Activity (RBA). The highest RBA value in this study was found in treatment C then treatment B with 30 days feeding period. Furthermore, treatments C and B also gave a higher RBA value than treatment A at time of feeding period 20 and 10 days. It can be concluded that the administration of probiotics in the form of *Bacillus subtilis* endospores in grouper can increase the RBA value of grouper (*Epinephelus coioides*). The best treatment of the administration of probiotic *Bacillus subtilis* endospore for the grouper (*Epinephelus coioides*) was 1 %. The best feeding period for the grouper (*Epinephelus coioides*) is at 30 days of feeding (Table 1).

Table 1. Leukocytes Respiratory Burst Activity (RBA) in Grouper (*Epinephelus coioides*)

Feeding Administration (Day)	Feed (Probiotic *Bacillus subtilis* Endospores)		
	0%(A)	0.1%(B)	1%(C)
10	1.87^{a}	1.91^{b}	1.98^{b}
20	1.83^{a}	2.12^{b}	2.12^{b}
30	1.88^{a}	3.52^{c}	5.03^{d}

* Mean significantly different (P<0.05).

d. Leukocyte Proliferation of Grouper (*Epinephelus coioides*)

Feeding probiotic *Bacilius subtilis* did not significantly affect the leukocyte proliferation value. The highest leukocyte proliferation value in this study was found in treatment C with reared period 20 days. While the lowest leukocyte proliferation value in this study was found in treatment A with reared time 30 days (Table 2). It can be concluded that feeding probiotic *Bacillus subtilis* endospore to the grouper does not have a significant effect on the leukocyte proliferation value of the grouper (*Epinephelus coioides*).

Table 2. Leukocyte proliferation in grouper (*Epinephelus coioides*)

Feeding Administration (day)	Diet (Probiotic *Bacillus subtilis*)		
	0%	0.1%	1%
10	1.31	1.25	1.10
20	1.50	1.59	1.88
30	1.10	1.21	1.21

*Mean do not vary significantly (P>0.05).

2. Discussion

The total numbers of the bacteria in rearing media for the treatment A (0 % *Bacillus subtilis*) had the lowest total number compared to other treatments for elapsed time 10 days, 20 days and 30 days sampling. Meanwhile, on treatment B (0.1% *Bacillus subtilis*) on the 30 days of sampling had the highest total number of bacteria in the rearing media compared to other treatments. While the treatment of feed C (1% *Bacillus subtilis*) had the highest total number of bacteria in the rearing media at the sampling time of the 10th and 20th days sampling. An increasing in the number of bacteria in treatments B and C indicated a better rearing media for the growth of the grouper, this is in line with (Ferdynan Sumule & Trisnawati Tobigo, 2017) which states that the administration of probiotics in the rearing media can improve environmental quality so that it has a significant effect on growth of red tilapia (*Orechromis sp.)*. With an increase in the number of bacteria in the environment of fish rearing, especially in the presence of Bacillus subtilis, this is as stated by (Olmos et al., 2019) that the administration of probiotics has a 95% positive impact through increasing nutrient absorption and assimilation, preventing disease development and increasing environmental parameters.

Administration of the probiotic *Bacillus subtilis* did not significantly affect the body weight of the grouper (*Epinephelus coioides*) because of the short rearing period(only 30 days). This is different from the research of (Ferdynan Sumule & Trisnawati Tobigo, 2017) and (Nair et al., 2021)which states that the administration of probiotics has a very significant effect on the growth of tilapia (*Oreochromis sp).* and *Etroplus suratensis*.This difference is due to the fact that tilapia (*Oreochromis sp)* and *Etroplus suratensis* are fast-growing fish, while the grouper is a slow-growing fish. So to get probiotics that have a significant effect on body weight of the grouper, a longer maintenance time is needed.

In this study, Treatment C (1 % Probiotic *Bacillus subtilis*) with a feeding period of 30 days obtained the highest RBA value 5.03 ± 0.02 compared to other treatments, so it can be concluded that in treatment C (1 % Probiotic *Bacillus subtilis*) with feeding period for 30 days had better immune resistance than other treatments. This result also in line with (Liu et al., 2017) that *Bacillus subtilis* E20 was improved innate immunity of O*plegnathus fasciatus*. The higher of RBA value indicated that higher level of fish immune system because the RBA value is the basic building block of the antibacterial system in fish. Increased RBA values can be correlated with increased phagocytic cell activity (Rawling et al., 2012). Respiratory burst can increase oxygen consumption to carry out phagophytic activity (Rieger & Barreda, 2011).

The highest leucocyte proliferation value in this study was found in treatment C with elapsed time 20 days. While the lowest leucocyte proliferation value in this study was found in treatment A with elapsed time 30 days (Table 2). Based on these results, it can be concluded that feeding probiotic *Bacillus subtilis* to the grouper does not have a significant effect on the leucocyte proliferation value of the grouper (*Epinephelus coioides*). This can occur because the increase in leukocytes usually occurs in fish that are under stress. While at the time of treatment the fish did not experience. According to (Sakai, 1999) states that stress interferes with non-specific immune responses such as an increase in lymphocytes.

Conclusion

The results of the research can be conclude that the administration of probiotics in the grouper (*Epinehelus coioides*) feed with the probiotic dose of *Bacillus subtilis* in treatment B (0.1% *Bacillus subtilis*) and treatment C (1 % *Bacillus subtilis*) had a significantly effect on Leukocytes Respiratory Burst Activity (RBA) compared to treatment A (0% *Bacillus subtilis*). The RBA values in treatment B (1.91) and treatment C (1.98) were significantly

different start from 10 days of rearing time. While the best dose for the RBA value is treatment C (5.03) with an elapsed time 30 days.

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