Effects of encapsulated *Bacillus* sp. D2.2 on gut bacterial composition and immune system in brown-marbled grouper *Epinephelus fuscoguttatus*

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**Abstract.** In the aquaculture industry, the application of probiotics is well known widely used to control disease, improve water quality and reduce demands for the use of antibiotics or disinfectants. However, some local gut bacteria can bind to harmful bacterial component. For better efficacy of probiotics in the digestive tract of carnivorous fish, encapsulation can be a simple, harmless, and improved method to maintain the microflora of the digestive tract thereby enhancing the immune system of fish. This study was conducted to investigate the effects of encapsulated probiotic *Bacillus* sp. D2.2 on gut bacterial communities and immune system in brown-marbled grouper *Epinephelus fuscoguttatus*. One hundred fish weighing about 40 g were divided randomly into five groups including negative control and positive control groups which were fed by a commercial diet only and a diet containing probiotic respectively, the third to fifth groups were fed by 1, 2, and 3 g/kg encapsulated probiotics dose of feed respectively. The results showed that encapsulated probiotic increase viability of probiotic bacteria and also affect the abundance of lactic acid bacteria. Immune response of the brown-marbled grouper also increased significantly after the application of encapsulated probiotics.

1. **Introduction**

The rapid development of aquaculture related to efforts to meet the world's food needs often encounter obstacles such as various diseases, survival, reproduction, and decreased water quality. The eco-friendly approaches to aquaculture are currently getting more attention because they ensure the sustainability of aquaculture practices. Microbes have been evaluated as probiotics in aquaculture which are components of living, dead or microbial cells that are applied in feed and water that are beneficial to the host [1]. The application of probiotics can reduce the use of antibiotics, chemicals or medicines that have the risk of increasing accumulation, biomagnification and resistance to pathogens [2]. Probiotics have many advantages such as enhancing growth performance, optimizing feed, increasing disease resistance, as well as improving stress and tolerance response [3].

Persistent microbiota usually colonize exposed body surfaces of the hosts such as skin, gills and digestive tract and are covered by a protective mucus that prevents the entry of harmful microbes [4]. However, due to the constant intake of food, water and microorganisms contained therein by aquatic
organisms, the water flowing through the digestive tract affects the microbiota in their intestines [3] either temporarily or permanently. The microbiota could be a combination of potentially pathogenic bacteria such as *Salmonella*, *Listeria*, and *Escherichia coli* [4], probiotics such as *Bacillus*, *Enterococcus*, *Lactobacillus* [5] and other microorganisms such as facultative anaerobic *Vibrio*, *Pseudomonas* [6], as well as certain yeasts and protozoans [7]. The composition of the gut microbiota is also known to be influenced by the feeding habits of the host [8]. Carnivorous fish tend to have lower gut microbiota diversity compared to omnivores and herbivores [9, 10]. Furthermore, at the same trophic level but different fish species may result in variations in gut microbiota composition which may be influenced by species-specific diets [11].

The application of probiotics in aquaculture considers their ability to inhibit or eliminate pathogenic bacteria. Such prophylactic treatment has been shown to increase resistance to pathogens and has been shown to increase the survival of several aquaculture species such as tilapia [12], carp [13, 14, 15], rainbow trout [16] and cod [17]. Mainly probiotics that aim to improve their performance in the intestinal tract are given orally through feed. Gastrointestinal conditions in carnivorous fish generally have a lower pH than herbivores, while in order to work optimally, probiotics must be maintained and stable during the administration system [4]. In an effort to optimize the work of probiotics, especially in the gastrointestinal tract, encapsulation of probiotic feed is a good way of delivery. Encapsulation is a coating process of a core material, in this case probiotic bacteria, using certain encapsulated materials that useful for maintaining survival and protecting probiotic bacteria from deterioration due to environmental unfavorable conditions, such as heat, chemicals, acids, stomach, and bile salts [18]. A research [19] revealed that feed encapsulation is efficient because it can ensure the delivery of the desired quantity, protects the probiotics from the harsh gastric environment, prevents deteriorate by water and temperature and is also effective in transporting them to the intestines. Proteins as well as carbohydrates are the most common materials used as carrier in encapsulated probiotic bacteria because their glassy state at storage temperatures ensures minimizing molecular mobility and therefore degradation [20].

In this study, *Bacillus* sp. D2.2, a local probiotic that has the potential to increase growth and immunity in fish and shrimp [21-26] was used. This probiotic was given in the form of feed encapsulation. Its effect on the bacterial community in the gastrointestinal tract and the immune system of the carnivorous brown-marbled grouper *Epinephelus fuscoguttatus* was investigated.

2. Materials and Methods

2.1. Bacterial culture

All *Bacillus* sp. D2.2 cultures were grown in seawater complete (SWC) agar. SWC agar comprised of peptone (Merck) 5 g, yeast extract (Merck) 1 g, glycerol (Merck) 3 ml, 75% sterile seawater 1000 ml, and 15 g agar (Merck) while SWC broth without agar. The SWC agar plates added by rifampicin 50 μg ml⁻¹ were inoculated with *Bacillus* sp. D2.2 aseptically and incubated for 24 h at room temperature (28-31°C). *Bacillus* sp. D2.2 isolates grown on the plates were isolates resistant to rifampicin. Thus rifampicin was used as marker of probiotic *Bacillus* sp. D2.2. Afterwards, isolates were transferred into 500 ml SWC broth and incubated in water-bath shaker for 140 rpm at room temperature for 18 h. SWC broth culture was then centrifuged at 5000 g for 20 min. Centrifuged *Bacillus* sp. D2.2 pellet was suspended twice in phosphate buffer saline (PBS) and then was counted to determine colony-forming units (CFU) ml⁻¹ of probiotic in agar medium.

2.2. Encapsulation of *Bacillus* sp. D2.2

*Bacillus* sp. D2.2 encapsulates were prepared using a method according to [18, 27] with modifications. Briefly, 10% (w/v) skimmed milk powder and 20% (w/v) sterile maltodextrin were homogenized with 10⁸ CFU ml⁻¹ suspension of *Bacillus* sp. D2.2 and frozen at -40°C. The frozen suspension of skimmed
milk powder-maltodextrin mixed with Bacillus sp. D2.2 was then freeze-dried (Labfreez FD-10-MR) for 36 h. The encapsulation probiotic were packed at storage temperature for feed treatments.

2.3. Feeding treatments
Commercial feed (protein 45%, fat 10%, ash 13%, moisture 10%, fiber 2%) were mixed with 6% encapsulated probiotic by re-pelleting. There were three feeding treatments, namely 1, 2 and 3 g of encapsulated probiotic feed per kg of commercial feed (treatment A, B, and C). In addition, there was a positive control (K+) where 6% probiotic was given without the encapsulation process and a negative control (K-) where the feed given was merely commercial feed.

2.4. Fish rearing conditions
The brown-marbled groupers with an initial average weight of 47±2 g were reared in a seawater container with a volume of 50 liters. The stocking density was 20 fish per container with 3 replications for each treatment. The fish were acclimated for a week before treatment. Fish were reared for 30 days and fed 3 times a day with 2% feeding rate under optimal water quality conditions, that dissolved oxygen > 5 ppm, temperature at 27-30 °C, pH of 7 - 8.5, salinity at 27-34 ppt, and total ammonia nitrogen (TAN) < 0.3 ppm [28].

2.5. Challenge test
The brown-marbled groupers that had been treated for 30 days were then challenge on the 31st day by injecting 0.1 ml the pathogenic bacteria Vibrio alginolyticus 10⁷ CFU ml⁻¹ intra-peritoneally. Survival rate (SR) was calculated for each treatment as formulated:

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SR (%) = \left( \frac{\text{the number of fish that live at the end of the study}}{\text{the number of fish that live at the beginning of the study}} \right) \times 100
\]

2.6. Parameter observations
Viability assessment was conducted to determine the comparison of the number of bacteria before and after the encapsulation process. High viability indicates the ability or viability of probiotic bacterial cells to grow normally under optimal conditions. Viability of initial CFU ml⁻¹ of Bacillus sp. D2.2 probiotic was conducted prior treatments in agar medium. In addition, Bacillus sp. D2.2 probiotic viability after encapsulated treatments and orally administration was enumerated by isolating intestinal bacteria using total plate count (TPC) method in SWC agar enriched by rifampicin. Oral administration followed the feeding treatment design which was 1, 2 and 3 g of encapsulated probiotic feed per kg of commercial feed including a positive and a negative control.

Density of lactic acid bacteria was observed by taking 1 g of fish intestine to be suspended in 9 ml sterile seawater and then a serial dilution was conducted. As many as 25 μl of suspension was inoculated in de Man-Rogosa-Sharpe Agar (MRSA) medium (Merck). Similarly, 25 μl of gut bacterial suspension was inoculated in tryptic soy agar (TSA, Merck) in order to grow other bacterial communities. After 24 h incubation at room temperature, the lactic acid bacterial cells and other bacterial cells were enumerated in CFU ml⁻¹. Identification of dominant bacterial community was conducted by using Microbact-24E (Oxoid).

On days 0, 30 and 37 of experiment, blood samples were collected from each treatment fish in a syringe rinsed with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. To obtain serum, blood was drawn using a syringe that was not rinsed with anticoagulant. Immunological assays was examined including erythrocyte count (EC) and leukocyte count (LC), as well as differential leukocyte count, i.e. lymphocyte, monocyte and neutrophil that were counted manually using Neubauer hemocytometer slide [29].
2.7. Statistical analysis
Quantitative results are expressed as the mean values ± standard deviation (SD). Differences between treatments were determined using a one-way analysis of variance and the significance of the difference between means was determined by Duncan’s multiple range tests with the statistical software package SPSS. Significant differences were accepted at P < 0.05.

3. Results and Discussion

3.1. Viability assessment
The viability of Bacillus sp. D2.2 probiotic before treatment was recorded as $9.7 \times 10^8$ CFU ml$^{-1}$. After encapsulation and oral administration, the viability of Bacillus sp. D2.2 probiotic was significantly decrease. The survival rate of Bacillus sp. D2.2 after encapsulation and oral administration remained low at 10% in all encapsulated treatments. However, without encapsulation, probiotic cells showed less survival than other encapsulation treatments, with only 0.9% of survival rate remain. This suggested that encapsulation technique with 10% (w/v) skimmed milk powder and 20% (w/v) maltodextrin may provide better protection for probiotic from digestive process. Among the various doses of encapsulated probiotics, the number of probiotic cells showed similarity (Table 1). The decrease in viability after encapsulation may also indicates the effect of cold temperatures during the freeze-drying process. Although the survival rate of the encapsulated probiotics was low after oral administration, the bacterial density was still maintained at the level of $10^8$ CFU ml$^{-1}$. Previous studies also confirmed the use of probiotics was maintained at a density level of $10^6-10^{10}$ CFU ml$^{-1}$ [30, 31].

| Feeding treatment | Prior encapsulation (CFU ml$^{-1}$) | After encapsulation and oral administration (CFU ml$^{-1}$) |
|-------------------|-----------------------------------|----------------------------------------------------------|
| 1 g kg$^{-1}$ (A) | $9.7 \times 10^8$                  | $1.02 \times 10^8 \pm 5.5^{ab}$                          |
| 2 g kg$^{-1}$ (B) | $9.7 \times 10^8$                  | $1.13 \times 10^8 \pm 7.2^{bc}$                          |
| 3 g kg$^{-1}$ (C) | $9.7 \times 10^8$                  | $1.20 \times 10^8 \pm 1.5^c$                            |
| without encapsulation (K+) | $9.7 \times 10^8$ | $9.40 \times 10^6 \pm 9.5^a$                            |

The bacterial composition observed before treatment showed the dominance of Vibrio species in the fish gut. The identification results showed that two Vibrio strains dominated the digestive tract of the brown-marbled grouper. Vibrio alginolyticus has been recognized as one of the most pathogenic marine bacteria that can infect marine organisms and humans. This species is found in its natural habitat in coastal and marine waters as well as fish and seafood. Its ability to carry pathogenic genes makes it an emerging pathogen of foodborne diseases [32]. V. furnissii which is related to V. fluvialis [33], is a pathogen that often contaminates seafood [34] and infects marine organisms such as shrimp [35], shellfish [36], and ornamental fish [37]. This species is also known as a zoonotic agent that can cause disease in animals and humans [38]. Even this species is reported to be resistant to antibiotics [39]. Observation of the bacteria in the intestines of fish at the end of the study showed a change composition where Vibrio no longer dominated the intestines of fish that received probiotic treatments, either encapsulated or not. Vibrio only dominated the intestines of fish in negative control that did not receive probiotic treatment in their feed (Table 2).
Table 2. Dominant bacterial composition in gut of brown-marbled groupers

| Feeding treatment | Prior feed treatment | After feed treatment |
|-------------------|----------------------|----------------------|
| 1 g kg⁻¹ (A)      | *V. alginolyticus*    | *Bacillus subtilis, B. brevis* |
| 2 g kg⁻¹ (B)      | *V. furnissii, V. alginolyticus* | *B. subtilis, B. brevis* |
| 3 g kg⁻¹ (C)      | *V. furnissii*        | *B. subtilis, B. brevis* |
| without encapsulation (K+) | *V. furnissii, V. alginolyticus* | *B. subtilis, B. brevis* |
| commercial feed (K-) | *V. furnissii, V. alginolyticus* | *V. furnissii, V. alginolyticus* |

The identified *Vibrio* strains indicate a possible pathogen. *V. alginolyticus* is a major fish pathogen within the genus *Vibrio* and has caused massive mortality since 1980’s [40]. Although several studies have reported the use of *V. alginolyticus* as a potential probiotic [41, 42], mass mortality is still common, as in grouper hybrids involving mixed infections of *V. alginolyticus*, *V. harveyi* and *Streptococcus iniae* [43]. Mostly *V. alginolyticus* together with *V. anguillarum* are known as the main type of *Vibrio* that infects groupers [44]. *V. furnissii* belongs to the second batch of *Vibrio* species reported to be pathogenic since the 1990s, infects European eel *Anguilla anguilla* and has been reported to be associated with epizootic diseases of various fish species [40].

In aquaculture, *Vibrio* species are categorized as opportunistic pathogens. *Vibrio*’s opportunistic nature is related to its virulence due to its cellular elements. In particular, *Vibrio* possesses virulence-related genes commonly found in its mobile genetic elements such as integrative and conjugative elements, genetic island, prophages, transposons, and plasmids [45]. These elements will result in the emergence of virulent strains even in non-pathogenic strains [46; 47], making *Vibrio* sp. as an opportunistic pathogens.

Probiotic candidates generally come from the lactic acid bacteria (LAB) group. LAB such as *Lactobacillus* or *Bifidobacteria* are probiotics that inhabit the digestive tract so that they are tolerant of the acidic and bile environment [48]. In addition to LAB, *Bacillus* spp. is another probiotic that can be active in wider range of environmental conditions [49], has the ability of adhesion, produces bacteriocin and is able to stimulate the immune system [48]. In this study, the administration of probiotics until the end of the study was able to increase the abundance of LAB regardless of whether it was encapsulated or not (Table 3).

Table 3. Density of lactic acid bacteria in gut of brown-marbled groupers

| Feed treatment       | Prior feed treatment (x 10⁸ CFU ml⁻¹) | After feed treatment (x 10⁸ CFU ml⁻¹) |
|----------------------|--------------------------------------|-------------------------------------|
| 1 g kg⁻¹ (A)         | 10.8 ± 1.6 ¹                        | 13.2 ± 6.1 ²                       |
| 2 g kg⁻¹ (B)         | 10.6 ± 1.0 ¹                        | 13.7 ± 4.0 ²c                      |
| 3 g kg⁻¹ (C)         | 10.3 ± 1.5 ¹                        | 14.3 ± 4.0 ³                       |
| without encapsulation (K+) | 10.3 ± 9.5 ¹                        | 12.9 ± 5.8 ²b                      |
| commercial feed (K-) | 10.5 ± 1.0 ¹                        | 10.7 ± 1.0 ¹                       |

LAB strains have the ability to produce lactic acid which partially inhibits the growth of pathogenic bacteria and strengthens their mucosal barriers [4] making them suitable to function as probiotics. Mucus is a gel-like layer that functions as a pathogen barrier [50]. The presence of probiotics in the digestive tract of fish can lead to competitive exclusion of pathogenic bacteria because these probiotics will compete with pathogens for nutrients, oxygen, and binding sites to attach to mucus or epithelial...
surfaces [4]. In a study showed that the inhibition of pathogens by *Bacillus* spp. not caused by the production of antibiotic components but iron deficiency conditions. *Bacillus* species is a probiotic producing sidherophore that will auto-compete pathogens by removing them from available iron ions [48].

3.2. *Immunological assays*

Observation of the EC and LC was carried out to determine the response of erythrocyte cells to the presence of antigens. In this study, the EC and LC of brown-marbled groupers increased after 30 days of feeding treatment (Figure 1a, b). It indicates that erythrocytes and leucocytes provided a higher response to the administration of encapsulated probiotics, especially at doses of 2 and 3 g kg\(^{-1}\) compared to controls. The number of erythrocytes after 30 days of the probiotic treatments ranged from 0.96±0.07 to 1.17±0.08x10\(^6\) cells mm\(^{-3}\) while leukocytes ranged from 1.51±0.03 to 1.78±0.04x10\(^5\) cells mm\(^{-3}\).

Furthermore, post-challenge test (day 37) with pathogenic bacteria, erythrocytes and leucocytes in the probiotic treatments, either encapsulated or not, showed higher responses than the negative control. After challenge test, the EC of the probiotic treatment was recorded in the range of 1.24±0.16 to 1.65±0.14x10\(^6\) cells mm\(^{-3}\) while the LC ranged from 1.74±0.02 to 1.95±0.03x10\(^6\) cells mm\(^{-3}\). While the EC in K- tended not to increase and was stable in the range of 0.77±0.06 to 0.98±0.07 08x10\(^6\) cells mm\(^{-3}\) (Figure 1a) and LC ranged from 1.39±0.01 to 1.46±0.05x10\(^5\) cells mm\(^{-3}\) throughout the observation time (Figure 1b).

Normal EC in marine teleostei vary as in rainbow trout (*Salmo gairdneri*) 0.77-1.67x10\(^6\) cells mm\(^{-3}\) [51] and striped hybrid bass 3.66-4.96x10\(^6\) cells mm\(^{-3}\) [52]. Similarly, leukocytes exhibit a wide variation in number among fish species. In marine habitat, golden trout had LC 0.21x10\(^5\) cells mm\(^{-3}\) [53] while in striped hybrid bass was reported in the range of 0.32 to 1.15x10\(^5\) cells mm\(^{-3}\) [52].

EC, together with hemoglobin and hematocrit, is one of important parameters that indicate anemia in fish. The normal range and no EC fluctuations in marbled grouper during the study showed that the fish were not anemic [31]. Leukocytes also play an important role in non-specific immunity, because they respond to incoming antigens mainly through the process of phagocytosis. Increased LC at the time of probiotic administration and challenge test with pathogenic bacteria showed a good leukocyte response to the antigens. The EC and LC conditions on K- which remained low showed that without probiotic administration, the hematological response was not properly triggered.

**Figure 1.** Erythrocyte (a) and leucocyte (b) counts of brown-marbled groupers after challenge test. Different letters on each bar (mean±SD) indicated significant differences (Duncan p<0.05). Feed treatment without probiotic (K-), feed treatment without encapsulation (K+), administration of encapsulated probiotic in feed at dose of 1 g kg\(^{-1}\) (A), 2 g kg\(^{-1}\) (B), 3 g kg\(^{-1}\) (C).

The leukocyte differential in this study showed an interesting trend. Of all the differential leukocytes, the encapsulation of *Bacillus* sp. D2.2 was shown to be able to significantly improve non-
specific immune response compared to controls (Figure 2a, b, c). The percentage of lymphocytes at the beginning of the study was in the range of 40%, the most compared between monocytes and neutrophils. On day 30, the lymphocyte percentage then decreased along with an increase in the probiotic encapsulation doses. This trend of decreasing lymphocyte percentage was more significant after the challenge test (Figure 2a). Lymphocytes are the most commonly observed leukocytes in fish peripheral blood films. Typically, lymphocytes represent 60-80% of leukocyte differential; such as in Siberian sturgeon *Acipenser baerii* supplemented with *Lactobacillus plantarum* was in range of 80% [54]. However, in this study, the range of lymphocyte percentage after treatment was 20-30%. This condition is in line with the study of *Inonotus obliquus* enriched diet administration on the kelp grouper (*Epinephelus bruneus*) [55]. Lymphocytes are not primary phagocytic cells like monocytes and neutrophils. The decrease in the percentage of lymphocytes is also related to its main function in triggering a specific immune response. Lymphocytes play an important role in the humoral and cellular immunity of fish. B lymphocytes in teleost fish function in the same way as mammalian B-1 cells and produce immunoglobulin M (IgM), triggers the formation of antibodies [29].

Monocytes are active phagocytic cells that will participate in the inflammatory response in fish [56]. Piscine monocytes occur in low numbers, less than 5% of the leukocyte differential [29]. However, research conducted by [55] showed a range of monocytes above 5%. Our research showed similar result where the monocytes of marbled grouper were in the range of 30-40% leukocyte differential (Figure 2b), indicated that there were more phagocytic cells in the blood. This is reinforced by the results showing an increase in monocytes after administration of probiotics and after the challenge test.

During infection, phagocytic cells will eliminate the incoming antigen. As part of the granulocytes, neutrophils are found in small numbers, but they are at the forefront when inflammation occurs. This could be seen from the increase in the percentage of neutrophils that occurred in the administration of probiotics and after the challenge test (Figure 2c). In early inflammation, neutrophils have an important role in non-specific immunity, not only phagocytosis, but also intracellular killing and digestion of phagocytic organisms [29].

![Figure 2](image-url)

*Figure 2.* Percentage of lymphocyte (a), monocyte (b) and neutrophil (c) of brown-marbled groupers after challenge test. Different letters on each bar (mean±SD) indicated significant differences (Duncan p<0.05). Feed treatment without probiotic (K-), feed treatment without encapsulation (K+), administration of encapsulated probiotic in feed at dose of 1 g kg⁻¹ (A), 2 g kg⁻¹ (B), 3 g kg⁻¹ (C)
After the challenge test, the survival rate of brown-marbled groupers given encapsulated probiotics was in the range of 70%. Encapsulation probiotic treatment with a dose of 3 g kg\(^{-1}\) was able to increase significantly the survival rate up to 80% compared to SR treatment of commercial feed which was only 67% (Figure 3). The low mortality rate in the treatment after the challenge test indicated that the probiotics were not harmful to the survival of the fish but also indicated the possibility of low virulence of the pathogenic bacteria.

![Figure 3. Survival rate of brown-marbled groupers after challenge test. Different letters on each bar (mean±SD) indicated significant differences (Duncan p<0.05). Feed treatment without probiotic (K-), feed treatment without encapsulation (K+), administration of encapsulated probiotic in feed at dose of 1 g kg\(^{-1}\) (A), 2 g kg\(^{-1}\) (B), 3 g kg\(^{-1}\) (C)](image)

4. Conclusion
The administration of encapsulated probiotic help to recover the hematological parameter changes due to infection and improved non-specific immune response. Administration of encapsulated probiotic at dose 3 g kg\(^{-1}\) was effective to control Vibrio alginolyticus after challenge test result in higher survival rate and better hematological parameter values.

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