The Addition of Immunostimulant in Formula Feed to Improve Immunity and Growth of Juvenile Cobia (Rachycentron canadum)

Suryadi Saputra, Arief Rahman Rivaie, Silfester Basi Dhoe and Herno Minjoyo

Lampung Main Center of Marine Aquaculture, Directorate General of Aquaculture, Ministry of Marine Affairs and Fisheries, Republic of Indonesia
Corresponding author: suryadi.saputra@kkp.go.id

Abstract

Suryadi Saputra, Arief Rahman Rivaie, Silfester Basi Dhoe and Herno Minjoyo. The Addition of Immunostimulant in Formula Feed to Improve Immunity and Growth of Juvenile Cobia (Rachycentron canadum). 2021. The aims of the experiment were to determine optimum dietary immunostimulant level in formula feed on nonspecific immune response and growth performance of juvenile cobia fish. Fish were kept in net cages (0.8 x 0.8 x 0.6 m$^3$) installed in fiberglass tanks which were completed with aeration and flow-through water system for 30 days. Design of the study was completely randomized design with 4 treatments, each consisting of 3 replications. Initial density was 50 fish for each replication with average body weight 4.69 g. The immunostimulant content used it was sourced from commercial products Nutricell. The formula feed (crude protein 46% and lipid 9%) containing three levels of immunostimulant additional levels, namely A (0.3%), B (0.5%), and as control, K (commercial feed as first control) and M (0.0% as second control without added immunostimulant. The data observed were growth performance which were consisted of absolute weight, feed intake, survival rate, specific growth rate, feed conversion rate and protein retention and mortality rate after challenge test with LD50 (1.0 x 10$^9$ CFU/ml) of pathogenic bacteria Vibrio alginolyticus. The growth performances showed that fish fed with addition of immunostimulant 0.3% (A) obtained higher absolute weight values and significantly different (p<0.05) from M, but not significantly different from K. Challenge test at the end of the study showed that treatment A had lower mortality compared to other treatments which was indicated had higher immunity level compare to other treatments. Based on the results above, it can be concluded that the formula feed with added immunostimulant 0.3% (0.7% total in the feed) showed the survival rate and optimal growth performance of juvenile cobia.

Keywords: feed, immunostimulant, immunity, growth

Introduction

Cobia Rachycentron canadum (Linnaeus, 1766) is a marine carnivorous fish species that was approved as one of the Indonesian commodities by the Minister of Marine Affairs and Fisheries in 2021. Because cobia is one of most important marine fish for future aquaculture production (Nhu et al., 2011). Fast growth rate, adaptability for captive
breeding, low cost of production, good meat quality and high market demand especially for sashimi industry are some of the attributes that make cobia an excellent species for aquaculture (Gopakumar and Nazar., 2012).

The success of the Lampung Main Center of Marine Aquaculture (MCMA) in the production of cobia fish has a positive impact on the potential and development of the commodity according to the fish seed formula feed. Meanwhile, formula feed needs to meet the demands for fish as a source of energy (Giri et al., 2007), maintain health conditions (Lall 2000) and order to maximize the growth potential (Raggi et al., 2018).

The nutritional composition of artificial feeds that are not balanced cause fish to experience malnutrition. This condition causes slow growth and a low defense system, therefore, the fish are easily infected by diseases such as the *Brookynella Hostilis* parasite, *Cryptocaryon Irritans*, and *Ichthyobodo* (McLean et al., 2008), and the bacterial disease such as *Vibrio Algynoliticus* (Rameshkumar et al., 2014), streptococcosis, *Vibrosis*, and Pasteurellosis (Kurniastuty et al., 2014). According to Rahayu et al. (1986), parasitic infections has caused mechanical damage to the fish body's organs, leading to disturbances in physiological processes. It also causes a decrease in growth which becomes an entry point for other disease agents (McLean et al., 2008) and lead to mass death when it is very severe.

Several efforts have been made to control the spread of disease, such as the use of chemicals. However, long-term use disturb the aquatic environment, pathogen resistance, and the health of people consuming the fish because it is affected by antibiotic residues (Alifudin, 2002). This makes it necessary to use preventive measures for effective management of the occurrence of infectious diseases. The measures can be taken by stimulating the fish's body-defense system using immunostimulant ingredients. Since immunostimulants do not leave residue in the fish body, it is safe for human health and the environment (Payung & Manoppo, 2015). Therefore, this research aims to determine the optimum levels of immunostimulants in formula feed compared to non-specific immune responses and the growth performance of *Rachycentron canadum* juvenile cobia.
Materials and Methods

Time and Place
The testing activity was carried out for one month from September 22 - October 21, 2020. It was continued with a challenge test from October 22 - November 4, 2020, in a controlled tank at the Lampung Main Center of Marine Aquaculture (MCMA). Meanwhile, proximate and blood analyzes were carried out at the Fish and Environmental Health Laboratory of Lampung MCMA.

Test fish dan Maintenance Tank
The test fish used were 600 juvenile cobia fish from hatcheries at MCMA Lampung with an initial body weight of 4.69 g and an average initial length of 11.26 cm/fish. Furthermore, the seeds were reared for 30 days in a net cages (0.8 x 0.8 x 0.6 m). Seeds are maintained with a stocking density of 50 fish/net cage on a 2 x 1 x 0.8 m fiber tank. In this research, the feed used was dry pellets and was given approximately the same energy content (isoenergy) and protein content (46%). The test feed is formula feed of MCMA formulation with addition of feed supplements Aquacell-GF which contains organic minerals Cu, Zn, Mn and immunostimulant in its formulation. Aquacell GF is a product of nutricell which produced in Indonesia and produced with raw materials European quality for boost immune system fish and shrimp bodies in aquaculture intensive. However, the test feed contained different immunostimulants, using the following treatments:
Treatment A: Formula feed with the addition of 0.03% immunostimulant
Treatment B: Formula feed with the addition of 0.05% immunostimulant
Treatment M: Formula feed without the addition of immunostimulants as a second control
Treatment K: Commercial feed as the first control.
The composition and results of independent feed proximate analysis of each treatment and control are shown in (Table 1).

Experimental Design
This research used a completely randomized design (CRD) with 4 treatments and 3 replications. The data were analyzed using ANOVA and further test using Tukey’s test with a 95% confidence interval. The data that has been obtained were tabulated and analyzed using the MS office 2019 excel program and SPSS 24.
Procedure for Testing and Data Collection

Before starting the test, the seeds were reared for 7 days in fiber tanks using mixed feeds, which consisted of commercial pellets and independent feed. This was conducted for weaning (adaptation to feed) because the test fish had been fed with commercial pellets. After the weaning process is completed, the total weight and length of the fish were measured to determine the initial weight and length of the seeds. Meanwhile, self-feeding is carried out twice a day with a dose until full (at satiation) with a frequency of twice a day at 08.00 and 14.00 WIB. The seeds were maintained with a flow-through system and siphoning was carried out one hour after the fish were fed to maintain water quality.

The sampling was carried out once a week by weighing the total weight of the fish population in each replication and measuring their 10% body length to determine the growth of the fish. The amount of feeding was also recorded daily to determine the amount of feed intake. Meanwhile, to determine the immunity of seeds given immunostimulants, sampling of non-specific immunity tests including total leukocyte test, phagocytic rate, and phagocytic index tests were carried out at the beginning, middle, and end of the research. After the rearing, a total of 30 fish were taken from each treatment to be challenged by injecting Vibrio Alginolyticus bacteria at a dose of 1.0 x 10^9 CFU/ml.

Growth parameters

The average body weight gain (ABGW) as (g/fish) was estimated according to the following equation: Final weight gain (g/fish) = Mean of weight (g) at the end of the experimental period – weight (g) at the beginning of the experimental period ; Feed conversion ratio (FCR) = Total feed fed (g/fish) / total wet weight gain (g/fish) ; Specific growth rate (SGR) = (ln W₁ – ln W₀) / T x 100 ; W₁: final weight ; W₀: initial weight T: time between W₁ and W₀

Immunity Parameters

Total Leukocytes

The parameters of the blood count observed were the total number of leukocytes taken from the anterior kidney and spleen. The organ was taken for the total leukocyte test because it is a hematopoietic tissue that produces and stores erythrocytes (red blood cells), leukocytes (white blood cells), and platelets. After the organs were removed, they were chopped in HBSS (Hanks Balanced Salt Solution) and filtered through a 100 m nylon filter. The filtering results were put into a tube containing percoll, centrifuged at a speed of 500 g and a temperature of 4°C for 40 minutes, and divided into three, namely erythrocytes, leukocytes, and platelets. Furthermore, the leukocytes were taken and put into 3 microtubes.
and centrifuged at 3000 rpm and a temperature of 4°C for 10 minutes. After centrifugation, the supernatant was removed and the HBSS was added then centrifuged again. The supernatant was later discarded, and the leukocyte pellets were put together in a microtube with an L-15 medium. Subsequently, leukocytes were counted with a microscope using a hemocytometer and their number was estimated using with formula: \[ \sum_{\text{leucocyte}} = \sum \text{average of leucocyte} \times 5 \times 10^4 \text{cell/ml} \] (Anderson and Siwicki, 1993)

**Phagocytic Rate and**

A total of 200 µl leukocyte suspension was taken to calculate the rate and phagocytic index. It was further placed in a glass slide, covered with a cover glass, allowed to stand for 90 minutes, and was rinsed with HBSS. Subsequently, 200 µl of latex beads suspension was added, allowed to stand for 30 minutes, and rinsed with HBSS. Meanwhile, the fixation of leukocyte suspension with 200 µl 100% methanol was left for 5 minutes, rinsed with ddH2O, and the staining process was continued using Giemsa. After drying, the preparations were observed under a microscope at 100 times magnification. The number of cells showing phagocytic activity (phagocytosis rate) was counted from 200 µl leukocytes observed and the phagocytosis activity (phagocytosis rate and index) was calculated with formula: phagocytic rate (%) = \[ \frac{\sum \text{phagocytosis cell}}{\sum \text{leucocyte cell}} \times 100 \] ; Phagocytosis Index = \[ \frac{\sum \text{Phagocytosis of latex beads}}{\sum \text{leucocyte cell}} \] (Anderson and Siwicki, 1993)

**Results and Discussion**

The results of independent feed testing with the addition of different immunostimulants stored in controlled tanks for 30 days are shown in table 2. In this research, it was discovered that the absolute weight (AW) of independent feed with the addition of 0.3% immunostimulant (A) was better and significantly different (p<0.05) compared to the control independent feed (M), however, it was not significantly different from the control commercial feed (K). The amount of feed intake (FI) in treatment A was lower and different from commercial feed controls, but the AW value was not significantly varied. Furthermore, the challenge test showed that treatment A fed with 0.3% immunostimulant feed had lower mortality than other treatments after injecting *Vibrio Alginolyticus* at a dose of 1.0 x 10⁹CFU/ml.

Table 2 and Figure 4 showed that the growth performance of cobia fish that consumes feed and 0.3% immunostimulant (A) experienced better growth. Meanwhile, slower growth was obtained in the independent control treatment (M) and treatments with the addition of 0.5% immunostimulants (B). This is similar to the results of Saputra (2016), where the
leukocyte phagocytic activity was better than feed with high immunostimulants but had a slower growth rate. Moyle & Cech (2004) also said that leukocytes are blood cells that play a role in the immune system. Moreover, white blood cells help rid the body of foreign substances by invading pathogens through the immune system and other responses.

The amount of feed consumed by cobia seeds in treatment A was lower and significantly different from the control. However, the addition of weight was not different, which indicated that the addition of immunostimulants can also reduce feed consumption without affecting absolute weight (AW), daily growth rate (SGR), and feed conversion (FCR). According to Cromwell (2001), the addition of Cu to feed helps growth because it has anti-microbial properties increases immune capacity (Dorton et al., 2003), and also promotes growth when used as supplement with Mn and Zn (Ahola et al., 2004: Pertiwi, 2018).

Based on the graph in Figure 6, the total leukocytes in treatments A and K (commercial feed control) increased from day 1 to day 30. This showed that there is an immune response for increasing resistance to disease. Hartoyo et al. (2015) stated that the function of leukocytes is to protect the body from pathogens by phagocytosis and producing antibodies. Meanwhile, the factors that determine the number of leukocytes include biological activity, environmental conditions, age, and feed.

According to Barman (2011), disease resistance occurs because the phagocytic rate carried out by leukocyte cells increases at the beginning of the stimulation of the immune system and gradually decreases. This pattern is a function of the increase in total leukocytes in each blood component such as lymphocytes, monocytes, and neutrophils.

The graph of the average percentage of the phagocytic index showed an increasing pattern of all treatments, except treatment B which decreased on day 30. The stable phagocytic index, which is the number of latex bead particles phagocytized by macrophages, also increased the results obtained in treatment A (0.3% addition of immunostimulants) from 1.3, 1.5 to 1.6%. This is in line with Panigrahi et al., (2005) which stated that the phagocytosis index in fish increases after something has been given to the body to trigger the immune responses. In this research, the increase in phagocytic index indicated that the immunostimulant added to the feed can respond to the immune system involving phagocytic cells (macrophages). Meanwhile, the index decreases when the phagocytic cells in the body can not be distinguished. Qomariyah et al., (2017) stated that the decrease in the phagocytic
index was due to phagocytic cells in the body that are not fully differentiated.

In this research, after observing growth, it was continued with a challenge test by injecting *Vibrio Alginolyticus* bacteria (Figure 9). In the challenge test, fish experienced general clinical symptoms such as cobia being attacked by pathogenic bacteria, covering all-black body colors, swimming with weak movements, staying at the bottom of the tub, also found wounds with peeling skin, which led to the death of the fish. The results showed that cobia fish in treatments A and K (control) had lower mortality compared to other treatments. This occurred in treatment A, where some fish have formed a non-specific immune response in their bodies and survive from attack by pathogenic bacteria. In line with the statement of Johnny et al., (2002) that to prevent the bacterial attack, immunostimulants are used which are prevent bacteria attack, increase non-specific immune responses, and phagocytic activity.

**Conclusion**

The results showed that formula feed-in treatment A with 0.3% immunostimulant (0.7% total in formula feed) gave the best growth performance in the breeding phase of cobia fish and had a higher survival rate than other treatments.

**References**


Johnny, F and Roza, D. 2002. Pengaruh penyuntikan imunostimulan...
The Addition of Immunostimulant in Formula Feed to Improve Immunity and Growth of Juvenile Cobia (Rachycentron canadum)


Pertiwi, R.T.A. 2018. The content of lead, cadmium, cuprum and zinc in anchovy (Stelophorus sp) and white shrimp (Penaeus merguensis) in Kao of North Halmahera. Aquasains 6 (2): 577-584


Table 1. Composition (% as fed) of formula feed for juvenile cobia

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Immunostimulant (%)</th>
<th>K (0,0)</th>
<th>M (0,0)</th>
<th>A (0.3)</th>
<th>B (0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>nk</td>
<td>42.80</td>
<td>42.80</td>
<td>42.80</td>
<td></td>
</tr>
<tr>
<td>Meat bone meal</td>
<td>nk</td>
<td>8.03</td>
<td>8.03</td>
<td>8.03</td>
<td></td>
</tr>
<tr>
<td>Poultry meat meal</td>
<td>nk</td>
<td>19.50</td>
<td>19.50</td>
<td>19.50</td>
<td></td>
</tr>
<tr>
<td>Soy bean meal</td>
<td>nk</td>
<td>6.10</td>
<td>6.10</td>
<td>6.10</td>
<td></td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>nk</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>Tapioca flour</td>
<td>nk</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>Wheat flour</td>
<td>nk</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td>Polar flour</td>
<td>nk</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>Oil fish</td>
<td>nk</td>
<td>6.60</td>
<td>6.30</td>
<td>6.10</td>
<td></td>
</tr>
<tr>
<td>Lecithin</td>
<td>nk</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>nk</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Premix vitamins</td>
<td>nk</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>nk</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Immunostimulant</td>
<td>nk</td>
<td></td>
<td></td>
<td>0.40</td>
<td>0.70</td>
</tr>
<tr>
<td>Anti mold</td>
<td>nk</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Anti oxidan</td>
<td>nk</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Trace mineral premix</td>
<td>nk</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Enzyme</td>
<td>nk</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Salt</td>
<td>nk</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>DL. Methionine</td>
<td>nk</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>nk</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

Analyzed composition

| Protein (%) | 46.12 | 46.0 | 46.0 | 46.3 |
| Lipid (%)   | 13.34 | 9.7  | 9.7  | 9.7  |
| Carbohydrate (%) | 19.82 | 21.6 | 21.6 | 22.5 |

Gross energy (kcal/kg) 4728.49 4456.4 4587.1 4510.7
C/P ratio 10.25 9.68 9.97 9.75

nk = not known

Tabel 2. Response of juvenile cobia fed formula feed containing different immunostimulant level for 30 days in tanks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( % Additional Immunostimulant</th>
<th>K (0.00)</th>
<th>M (0.0)</th>
<th>A (0.3)</th>
<th>B (0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Weight (g/fish)</td>
<td>26.22 ± 1.32</td>
<td>19.58 ± 3.01</td>
<td>23.91 ± 1.75</td>
<td>22.38 ± 1.35</td>
<td></td>
</tr>
<tr>
<td>Initial Length (cm)</td>
<td>11.44 ± 0.47</td>
<td>11.22 ± 0.68</td>
<td>11.14 ± 0.31</td>
<td>11.22 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Final Length (cm)</td>
<td>17.3 ± 0.14</td>
<td>15.91 ± 0.64</td>
<td>16.21 ± 0.20</td>
<td>15.19 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>Absolute Weight(g/fish)</td>
<td>21.47 ± 1.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.53 ± 3.30&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>18.54 ± 1.41&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.36 ± 2.76&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Feed Intake (g/day/fish)</td>
<td>1.23 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.77 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.78 ± 0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.65 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Survival Rate (%)</td>
<td>97 ± 3.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88 ± 4.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>92 ± 4.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>70 ± 12.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Specific Growth Rate (g/fish)</td>
<td>5.98 ± 0.31</td>
<td>5.05 ± 0.59</td>
<td>5.4 ± 0.19</td>
<td>5.26 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Feed Conversion Ratio</td>
<td>1.72 ± 0.09</td>
<td>1.63 ± 0.28</td>
<td>1.27 ± 0.23</td>
<td>1.32 ± 0.36</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> the same superscript letters on the same line show results that are not significantly different (P > 0.05).
The Addition of Immunostimulant in Formula Feed to Improve Immunity and Growth of Juvenile Cobia (Rachycentron canadum)

Figure 4. The graph of growth performance parameter of cobia fish at nursery phase that reared for 30 days (AW g/fish = Absolute Weight; SGR %/day = Specific Growth Rate; FCR = Feed Conversion Ratio; FI g/day/fish = Feed intake). (Treatment K: commercial feed as first control, M: the formula feed without added immunostimulant (0.0%) as second control, A: additional of immunostimulant 0.3% and B: additional of immunostimulant 0.5%).

Figure 5. Total leukocytes juvenile cobia fish. (Treatment K: commercial feed as first control, M: the formula feed without added immunostimulant (0.0%) as second control, A: additional of immunostimulant 0.3% and B: additional of immunostimulant 0.5%)
Figure 6. The Graph of phagocytic rate juvenile cobia fish. (Treatment K: commercial feed as first control, M: the formula feed without added immunostimulant (0.0%) as second control, A: additional of immunostimulant 0.3% and B: additional of immunostimulant 0.5%)

Figure 7. The Graph phagocytic index juvenile cobia fish. (Treatment K: commercial feed as first control, M: the formula feed without added immunostimulant (0.0%) as second control, A: additional of immunostimulant 0.3% and B: additional of immunostimulant 0.5%).
The Addition of Immunostimulant in Formula Feed to Improve Immunity and Growth of Juvenile Cobia (Rachycentron canadum)

Figure 8. The Graph of number of survivor fish after challenge test for juvenile cobia injected *Vibrio alginolyticus* for 14 days without feeding. (Treatment K: commercial feed as first control, M: the formula feed without added immunostimulant (0.0%) as second control, A: additional of immunostimulant 0.3% and B: additional of immunostimulant 0.5%).