Inclusions of probiotics and prebiotics in diet for coral trout, *Plectropomus leopardus* nursery

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**Abstract.** Coral trout is one of marine fishery commodities with high economic value. This grouper fish has limited ability in the utilization of artificial feed. The aim of this study was to obtain information on the effectiveness of probiotics and prebiotics inclusions in diet to increase growth of coral trout. Fish was reared in fiber tanks 300-L with a density of 30 fish/tank. Fish weight and length were 3.7±0.1 g and 6.3±0.1 cm, respectively. Each tank was equipped with a flowing water system and aeration as a source of oxygen supply. The feed was formulated with 48% protein and 9% lipid. Treatments were inclusion of probiotics in diet (diet A), inclusion of mannan oligosaccharide/MOS (diet B), inclusion of probiotic + mannan oligosaccharide/MOS (diet C), and diet D as control. The study was conducted in a complete randomized design (CRD) with 4 treatments and 4 replications. Fish fed trial diets with a frequency of 3 times a day at satiation. The parameters observed were growth, feed efficiency, feed digestibility, bacteria density and immunity. The data obtained were analysed with ANOVA followed by LSD. The results showed that diet with the probiotic content promoted better growth and feed efficiency of coral trout compared to the other treatments. The highest growth of coral trout fingerlings was obtained by fish fed diet A (added with probiotics) with a weight gain of 794.86%. While fish fed diet containing probiotics and prebiotic (diet C) showed a lower growth response (weight gain 641.26%) than those fed diet with probiotics and prebiotics (MOS) separately. Survival during the study for all treatments ranged from 90.00% to 93.33%.

1. **Introduction**

Coral trout, *Plectropomus leopardus* (Lacepède, 1802) is one of the high economic value marine fisheries commodities [1], thus the level of exploitation tends to increase. To prevent extinction due to over fishing, it is pivotal to develop coral trout aquaculture to supply the demand.

Research on coral trout aquaculture has been started in the Institute for Mariculture Research and Fisheries Extension in Bali since 2005 [2]. The fingerlings of the coral trout has been successfully produced from the hatchery [3], but the numbers are still very limited and the success of larvae rearing maintenance are highly fluctuated. For this reason, a deeper study is required to improve the success of fingerlings production. In general, there are several factors that influence the survival of fish including the environment, diet which includes natural and artificial feed, as well as diseases.

The provision of artificial diet for coral trout fingerlings still needs to be studied. Groupers are known to be able to consume and digest artificial diet [4]. Gultepe [5] reported that diet can improve growth performance, fish health and is an as important factor in the aquaculture industry. The ability
of fish to digest artificial diet is very dependent on the ability of the digestive tract of fish in breaking the bonds of nutrients present in artificial diet. To assist fish in optimizing the digestion of artificial diet, it can be done by adding probiotics in artificial diet. The use of probiotics from one type of Bacillus namely Bacillus cereus in artificial diet had been proved to produce higher growth in the coral trout fingerlings [6]. However, these results were not yet optimal so that trials of probiotics and prebiotics and symbiotics/uebiotics are still needed, particularly on a combination of probiotics and prebiotics in artificial diet for coral trout fingerlings.

Besides the use of probiotic bacteria in feed to increase the growth of coral trout, the dose of probiotic bacteria is a factor that needs to be taken into account in feed management because it plays an important role in the effectiveness of feed use. In the study of Fuller [7] reported that factors that influenced the host response to probiotics include: composition of host interstium microflora, dosage used, age and species or strain of host animal, quality of probiotics and ways of preparation of probiotics.

Probiotic bacteria are widely known as microbiological agents that are capable of producing extracellular enzymes. This ability is expected to assist fish in breaking complex bonds in the nutrients contained in artificial diet so that it can be maximally utilized by fish. Currently the use of probiotics in artificial diet is mostly performed by coating techniques or coating the surface of the diet with probiotics. This method is less efficient to implement since it requires additional time and energy. In addition, the addition of probiotics with coating techniques has a risk that the diet will be spoiled faster due to mould. Therefore, addition of probiotics or prebiotics in diet formulations might be the solution.

Probiotics are defined as living cells or substrates that provide benefits through growth stimulation, improved digestion, and a better immune response. Probiotics can also improve water quality [8]. In addition, Tuan [8] stated that several studies have shown that probiotics are beneficial for fish, such as growth stimulation to improve feed digestion, immune responses and water quality control.

Prebiotics are natural compounds in food that cannot be digested by the intestine, functioning as a supplement to encourage the growth of good microorganisms in the digestive system. One example is mannan oligosaccharide (MOS), which functions to improve intestinal function and microvilli integrity which results in greater absorption efficiency of the digestive tract [9]. Research on MOS administration by Pavličević [10] in rainbow trout (Oncorhynchus mykiss) showed the best growth results obtained by the lowest MOS level (0.05%), when compared to the control group and 0.1% and 0.15% MOS added to the feed. The growth increased by 11.5%. The best feed conversion ratio (FCR) of 1.29 was found with a MOS supplement of 0.05% compared to 1.54 in the control group. FCR both in 0.1% and 0.15% of MOS treatments were 1.46, also significantly better than in the control group. Moghaddam [11] stated the results of MOS administration on carp showed that oral administration of MOS at 0.20% improved the immune response, improved FCR and simple intestinal microbiota of carp.

The application of symbiotics is one of the biological control strategies that can increase the growth and disease resistance of aquaculture organisms [12]. Symbiotics are nutritional supplements that combine probiotics and prebiotics, so they can increase the beneficial effect on the host [12]. In this study symbiotics are a combination of probiotics and MOS prebiotics. Based on this, it is necessary to try research on the effectiveness of probiotics and prebiotics (MOS) added in formula diets. This study aimed to obtain information on the effectiveness of probiotics and prebiotics in increasing the growth of coral trout fish.

2. Materials and methods
The containers used in the experiment were 16 polycarbonate tanks with a volume of 300 litres. The tanks were equipped with a flowing water system and aerations as a source of oxygen supply. The fingerlings of coral trout were obtained from the hatchery, then the fingerlings were adapted to artificial feed until the fish responded to the feed properly. The coral trout fingerlings used had an average weight size of 3.7 ± 0.1 g and an average length of 6.3 ± 0.1 cm with a fish density of 30 fish/tank. Siphoning was conducted daily to remove sediments waste. The duration of the experiment
was 112 days. At the beginning and end of the experiment, length measurements were taken, and fish samples were collected for proximate analysis.

Experimental feed was formulated with 48% protein content and 9% lipid. Treatments were in the form of probiotic content in diet (diet A), MOS/Mannan oligosaccharide (diet B), probiotics + prebiotic MOS/Mannan oligosaccharide (diet C) and without probiotics and/or prebiotic MOS/Mannan oligosaccharide (diet D) as a control (Table 1). The study was designed in a completely randomized design (CRD) with 4 treatments and 4 replications. Fish fed 3 times a day ad satiation. To determine the response of the tested fish to the experimental feed, every 2 weeks the fish was weighed.

Table 1. Composition (g/100 g diet), proximate analysis of tested diets

| Ingredient                                | Tested diet |
|-------------------------------------------|-------------|
|                                           | A | B | C | D |
| Fish meal                                 | 49.1 | 49.1 | 49.1 | 49.1 |
| Squid liver meal                          | 12.0 | 12.0 | 12.0 | 12.0 |
| Krill meal                                | 10.0 | 10.0 | 10.0 | 10.0 |
| Soybean meal                              | 10.0 | 10.0 | 10.0 | 10.0 |
| Flour                                     | 5.8 | 5.8 | 5.8 | 5.8 |
| Vitamins Mix                              | 1.3 | 1.3 | 1.3 | 1.3 |
| Minerals Mix                              | 1.7 | 1.7 | 1.7 | 1.7 |
| Fish oil                                  | 2.48 | 2.48 | 2.48 | 2.48 |
| Probiotic                                 | 1.5 | 0.0 | 0.75 | 0.0 |
| MOS/Mannan Oligo Saccharide               | 0.0 | 1.5 | 0.75 | 0.0 |
| Taurine                                   | 0.5 | 0.5 | 0.5 | 0.5 |
| Astaxanthin                               | 0.1 | 0.1 | 0.1 | 0.1 |
| Carboxy Methyl Cellulose                  | 1.0 | 1.0 | 1.0 | 1.0 |
| Total                                     | 100 | 100 | 100 | 100 |

Proximate composition of tested diet:

| Ingredient       | Tested diet |
|------------------|-------------|
| Protein (% DM)   | 48.60 | 48.43 | 48.38 | 48.70 |
| Lipid (% DM)     | 9.59  | 9.48  | 9.48  | 10.46 |
| Ash (% DM)       | 16.36 | 16.05 | 15.81 | 15.79 |
| Fiber(% DM)      | 2.76  | 1.17  | 1.83  | 1.33  |
| Carbohydrate (% DM) | 22.69 | 24.87 | 24.50 | 23.72 |

In the second stage of the research, digestibility test was conducted using fish with a size of 20 g, as many as 30 fish/tank kept in 300-liter volume polycarbonate tanks. The tanks were equipped with aeration and running water systems. The diets used were the same as the feeds for growth test with an additional marker of 1% chromium oxide (Cr₂O₃) [13]. Fish fed 3 times a day at-satiation. Fish were adapted to the tested feed for the first 7 days. At day 8, fish faeces were collected shortly after the being expelled by the fish. Then the faeces were dried in an oven at 50-60°C. Dried faeces were then collected in closed containers. Stool collection was carried out until the amount was sufficient for
proximate and chromium analysis. Chromium concentration in feed and feces was analyzed according to the Takeuchi procedure [14]. The digestibility coefficient of dry matter and protein from the experimental feed was calculated according to Takeuchi [14] formula: ADC (%) = 100 x \( \frac{1 \times (MD \times AF)}{(MF \times AD)} \).

Where ADC = Apparent Digestibility Coefficient of dry matter or crude protein, MD and MF are chromium levels in feed and faeces, respectively. AD and AF are protein levels in feed and feces, respectively.

Proximate compositions of feed, fish body before and after the study were analysed using the AOAC method [15]. The data was analysed using a completely randomized design.

The parameters observed were final weight, specific growth rate (SGR), absolute growth (GR), weight gain (%), feed efficiency (EP), survival rate (S), total bacterial population, immunity test and digestibility of feed nutrients. Growth rates were calculated using the Heinsbrook formula and NRC:

Where SGR : Specific Growth Rate (%), GR : Absolute Growth (g), Wo : Fish weight at study entry (g), Wt : Weight of fish at the end of the study (g), t : Time (day)

Feeding efficiency (EP) was calculated using the following formula [16]:

\[
EP = \frac{(Wt + D - Wo)}{F} \times 100\%
\]

Where EP: Feeding efficiency (%), Wt : Fish biomass at the end of the study (g), Wo : Fish biomass at the beginning of the study (g), D : Fish biomass that died during the study (g), F : Amount of feed consumed during the study (g)

The degree of survival (survival) according to Effendi [17] was calculated by the formula:

\[
S = \frac{Nt}{No} \times 100\%
\]

Where S : Survival (%), Nt : The number of fish at the end of the study, No : The number of fish at the beginning of the study

Data then analysed with LSD with a confidence interval of 95% using IBM SPSS 20 for Windows to determine statistically difference.

3. Results and discussion

Based on the results of the study for 16 weeks (112 days), coral trout growth during the experiment is presented in Figure 1.

![Figure 1. Growth of coral trout during experiment](image)

Based on Figure 1, it shows that the highest increase in weight is seen in coral trout grouper which was fish fed diet A (probiotics), the second was that of fed diet D (control diet) and diet B (MOS).
Total weight gain of coral trout fed diet C (probiotics + MOS) was the lowest than other treatments. The addition of probiotics in fish has a working principle of utilizing microorganisms to break down or break down long chains of carbohydrates, proteins and lipids found in food. This happens because of the special enzymes that probiotics have. The breakdown of complex molecules makes it easy for fish to carry out chemical digestive processes (enzymatic reactions) and physics (peristalsis and absorption) in the digestive tract of fish.

The performance of the growth of coral trout grouper during the study is presented in Table 2.

**Table 2.** Performance parameters growth of coral trout grouper during experiment

| Treatment      | Parameter                         | Diet A (Probiotic) | Diet B (MOS) | Diet C (Probiotic+MOS) | Diet D (Control) |
|----------------|-----------------------------------|--------------------|--------------|------------------------|------------------|
|                | Average initial weight (g)        | 3.7±0.10a          | 3.7±0.14a    | 3.7±0.07a              | 3.7±0.17a        |
|                | Average final weight (g)          | 33.11±6.63a        | 27.92±7.26c  | 27.43±6.33c            | 30.20±5.43b      |
|                | Average initial length (cm)       | 6.3±0.10a          | 6.3±0.06a    | 6.3±0.06a              | 6.3±0.05a        |
|                | Average final length (cm)         | 12.73±0.32a        | 12.07±0.25c  | 11.97±0.49c            | 12.47±0.15b      |
|                | Absolute growth/W (g)             | 29.41±2.04a        | 24.23±0.60c  | 23.73±0.49c            | 26.50±0.96b      |
|                | Specific growth rate (%)          | 1.95±0.05a         | 1.81±0.05c   | 1.79±0.06c             | 1.88±0.06b       |
|                | Body weight gain/WG (%)           | 794.86±55.00a      | 654.79±16.24c| 641.26±65.46c          | 716.13±25.86b    |
|                | Feed efficiency                   | 80.32±0.04a        | 75.00±0.03c  | 67.64±0.07b            | 79.57±0.01a      |
|                | Nutrient digestibility value (%)  | 80.40              | 76.70        | 72.73                  | 79.74            |
|                | Survival rate (%)                 | 93.33±6.67a        | 93.33±5.77a  | 93.33±5.77a            | 90.00±3.33a      |

Table 2 shows that the average weight at the end of the study was significantly different. Specific growth rate (SGR) and absolute growth (GR) showed the highest growth obtained in fish fed diet A (Probiotic), diet D (control) and diet B (MOS) respectively. This is in accordance with research by Munir [18] which showed probiotic-based feed had more positive effect on growth, feed utilization and fish seed survival than feed with the addition of prebiotics. Growth trends are lower in all prebiotics compared to fish in probiotics [19]. Feed containing probiotics (diet A), growth control diets were higher than prebiotic MOS (diet B), or a combination of probiotics and prebiotic MOS (diet C). This indicates that administration of probiotics resulted in better growth of coral trout grouper, *Plectropomus leopardus* than those of prebiotic MOS or the combination of probiotics with prebiotic MOS.

The roles of probiotics in fish feed are as a competitor for pathogenic bacteria through habitat competition, nutritional competition and changes in the enzymatic activity of pathogens; a contributor for the availability of nutrients and increase digestibility of feed as well as more effective use of feed by contributing additional enzymes; direct absorption of bacteria from dissolved organic matter; increasing immune response to infectious pathogens; antiviral effects [20,21]. According to Marzuqi [22] positive proteolytic, lipolytic, and amylolytic testing of *Bacillus cereus* probiotics had the ability to break protein, fat, and starch bonding which could speed up metabolism in the digestive tract of coral trout, as a result their growth was faster. This is also supported by research by Faramazi [23].
which stated that increasing of growth and utilization of feed in fish were related to the increasing of nutrient digestibility. Most probiotics affect the digestive process through increasing the number and production of microbial enzymes, improving intestinal microbial balance, digestion, feed absorption and feed utilization [24]. Protease enzyme secretion can damage peptide bonds and produce free amino acids that can be absorbed by the host [24].

Meanwhile, probiotics play a role in increasing the activity of probiotics. Prebiotics are nondigestible food ingredients that are beneficial in stimulating the growth of bacteria that support health in the digestive tract to improve the balance of the population of host intestinal bacteria [25]. Intestinal probiotics hold an important position as producers of exogenous enzymes in the host. In accordance with the results of research by Sanjayasari [26] showed that pellets with probiotics had high performance on intestinal microflora and lipid deposition, with better feed efficiency. Welker [27] stated the use of MOS prebiotics in catfish (Ictalurus punctatus) showed an increase in growth rate, hematology, and immune function. However, when compared between probiotics and MOS prebiotics, it is better to use probiotics because they produce better growth. This is presumably because the addition of probiotics directly impacts the digestive system of fish by helping to provide additional digestive enzymes so that nutrients are more quickly absorbed. Meanwhile, MOS prebiotics when combined with probiotics had a negative impact for coral trout. Some studies also showed that the dose of probiotic and prebiotic administration in symbiotic applications could be one of the limiting factors to get optimal results on the host [12]. This showed that the administration of probiotics and prebiotics also needed to be sought for the best levels in order to obtain optimum results.

The efficiency of feed obtained in this study was ranged from 67.64 to 80.32%, based on the value of feed efficiency (EP) between diet A (probiotics) and diet D (Control) the percentage value of EP was not significantly different, but was higher than diet B (MOS) and diet C (probiotics + prebiotics). Data of this study showed the use of probiotics along with MOS type prebiotics inhibited growth and reduced the efficiency of feed absorption in tiger grouper. The highest value of feed efficiency found from fish fed with probiotics, which shown the highest growth. Digestion value of feed until the end of the study of each treatment ranged from 72.73 to 80.40%. This showed that the nutrients in the feed were utilized to the maximum which lead to the increasing growth, survival as well as stimulating digestion in the body of the fish.

The results of observations of red blood cells (total erythrocytes) and white blood cells (total leukocytes) of coral trout at the end of the study are presented in Figure 2 and Figure 3.

![Figure 2. Total of erythrocytes of coral trout](image)

Data of Figure 2 shows the highest number of erythrocytes in diet D (control) that is equal to 3.59 x 10⁶ cells / mm³, while the total erythrocytes produced by treatment A (probiotics) amounted to 1.31 x 10⁶ cells / mm³. However, this value is still in the normal range of the number of red blood cells in
fish, ranging from $1.05 \times 10^6$ cells / mm$^3$ [28,29]. Increasing the number of red blood cells is an attempt at homeostasis of fish body in an effort to increase hemoglobin to bind oxygen. Handling and anesthesia of fish in water without oxygen/aeration is one factor that can cause stress on the fish. This is supported by Wedemeyer & Yasutake [30] and Erika [31] which stated that the high total erythrocyte value in fish indicated that the fish was under stress. Fish which exposed to low oxygen water will experience hematopoiesis so that erythrocytes increase as an effort to adjust the addition of oxygen [32]. The number of erythrocytes in fish increases in an effort to reduce stress and adjust physiological conditions [33,34]. In addition, Erika [31] suggested that low total erythrocytes below the normal limit indicating that fish were anemic. The picture showed that an increase in the number of erythrocytes in the control treatment indicates that the fish is more susceptible to stress.

Figure 3. Total of leucocyte of coral trout

The first defense against disease in fish is carried out by leukocytes through phagocytosis, encapsulation and nodule formation [35]. The number of leukocytes in red spotted grouper reared in a temperature difference (25-34 °C) ranges from 8-14 x103 cells / mm$^3$ [36]. Figure 3 shows the lowest leukocyte count diet MOS which indicates that the fish is in good health. Diet D (control) showed the highest number of leukocytes that might indicate an infection. Infections occur in grouper aquaculture in North Bali are often parasitic infections. These parasitic infections often interfere with fish health so that they become susceptible to infections by other microorganisms such as viruses and bacteria. The parasite that often interferes with grouper aquaculture is leeches (hirudinea: Zelanycobdella arugamensis), although this parasitic infection does not result in death. However, the presence of parasitic infections can cause an increase in the number of blood leukocytes.

Addition of probiotics and prebiotics MOS into fish feed (diet C) results in lower growth than diet A and diet B, higher than diet D. It is suspected that the number of intestinal bacteria is very low due to the presence of prebiotic MOS in diet C (Figure 4). The low intestinal bacteria caused by *Bacillus cereus* as probiotics are not suitable when combined with MOS as prebiotics in feed for tiger grouper fish. The low number of intestinal bacteria causes growth is also lower, because there is an optimum number of probiotics for optimum fish digestion. [37] reported that the use of higher probiotic concentrations did not always lead to an increase in growth performance; there was an optimal concentration ratio between symbiotics (probiotics and prebiotics MOS) in order to obtain optimal growth.
The highest number of intestinal bacteria is shown in the control diet (Figure 4). This suggested that by combining probiotics with MOS, the number of probiotics was less than other feed treatments. Meanwhile, in feed with probiotics the number of intestinal bacteria was lower, this showed that the addition of probiotics suppressed some intestinal bacteria which might be pathogenic. Diet B (MOS) was lower than diet D (control) and diet A (probiotics), this was presumably because MOS had not been properly used either in quantity or levels of MOS against the intestinal bacteria of coral trout grouper. This was also shown in symbiotics in diet C (Probiotics + MOS), the lowest number of intestinal bacteria.

The survival rate during the study on coral trout fed with diet A (probiotic), diet B (MOS) and diet C showed the same survival rate and did not differ between treatments. This means that the feed added with probiotics or MOS still provided stronger survival. So that feed containing probiotics, prebiotic MOS, and symbiotics (probiotics + MOS) was still able to increase the survival of coral trout.

4. Conclusion
Based on the results of this study, it was concluded that feed with probiotic could increase growth and feed efficiency in the seeds of coral trout. The highest growth of coral trout fingerlings was obtained in feed containing probiotics in feed (diet A) with a weight gain of 794.86%. The use of probiotics plus prebiotics (MOS) in feed (diet C) provided a lower growth response compared to the use of probiotics and prebiotics (MOS) separately in diet with weight gain of 641.26%. It is recommended that feed formulation for coral trout should incorporate probiotics. Further research needs to be done to determine the levels of MOS prebiotics for coral trout in order to obtain optimal growth.

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