Utilization of a commercial probiotic, effective microorganisms, in diet fermentation for rabbitfish grow-out

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Abstract. Rabbitfish (Siganus guttatus) is a herbivorous fish that can utilize a plant-based diet better than omnivore and carnivorous fish. However, the quality of the plant-based diet still needs to be improved so that the rabbitfish can grow faster with better feed efficiency. One effort that is thought to be done to improve the quality of diet is fermentation. Therefore, this study aims to evaluate the use of effective commercial microorganisms (EM-4) probiotics in the fermented plant-based diet on the growth performance of rabbitfish. The treatments tested were fermentation of ingredient plant-based diet using EM-4 at different doses namely: F10 (10 mL), F20 (20 mL), F30 (30 mL), F40 (40 mL) per kilogram substrate and WF (unfermented diet). The test diets were given to the rabbitfish juvenile that was cultured in floating net cages in the sea. The initial weight of the test fish is around 48 g, maintained in 15 units of 1x1x1.5 m cage; they fed with satiation in the morning, afternoon, and evening for four months. The results showed that the fermented diet with EM-4 at all doses tends to increase in the content of free fatty acid, total n-3, total n-6, arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) compared to these fatty acids contents in the unfermented diet. The specific growth rate, weight gain, feed efficiency, and protein efficiency ratio was not significantly different (P>0.05) between the treatments. Feed intake was significantly different (P<0.05) between treatment, and it was highest in fish fed the unfermented diet compared to fish fed the fermented diet. Fermentation of the test diets with EM-4 has not been able to improve feed utilization and growth of rabbitfish.

1. Introduction
Rabbitfish (Siganus guttatus) are one of valuable fish which are in great demand in Indonesia in particular in South Sulawesi. Production of the seeds has been successful with small number juveniles to support grow-out trials in a floating sea cage. It is widely accepted that one of the main problems for fish culture is the cost of feed. According to Harris [1], the feed can contribute up to 70% of the total production costs in an intensive aquaculture system, including for rabbitfish grow-out. Therefore, it is necessary to look for relatively inexpensive feed for rabbitfish grow-out. In addition, commercial feed for rabbitfish has not been available so far.

A study reported by Parazo [2], juvenile rabbitfish require protein and energy approximately 35% and 3832 kcal/kg of feed, respectively, to grow optimally. This is a high protein content of feed for the herbivorous fish group. While protein is a component of nutrients that are quite expensive in fish feed. Therefore it is necessary to develop a feed for fish grow-out, which is inexpensive but still of high
quality to ensure optimum fish growth. One way to get cheaper feed is to utilize potential local raw materials that are abundant and have not been utilized optimally as well as improve their quality.

Although rabbitfish can utilize plant-based diet better than omnivorous and carnivorous fish, however, generally vegetable ingredient has high crude fiber content such as copra cake meal (15.4%–6.7%), sargassum meal (24.0–26.2%), and rice bran (6.2–14.3%), [3], and other anti-nutrient substances such as phytic acid on rice bran (10.0–10.1%), copra cake meal (0.26–0.33%), soybean meal (0.37–0.42%) [4], and galactomannan in copra cake meal (25%–30%) [5]. While the fish tend to not have or very low activity of cellulase enzymes and phytase enzymes in their intestines. Therefore, the quality of ingredients or plant-based feed needs to be improved. One effort to improve the quality of feed raw materials is through bioprocessing (fermentation).

To improve the quality of feed ingredients through bioprocessing, several types of microorganisms can be used, including those commercially available products. Effective microorganisms (EM) are commercial probiotics that are easily found in the market. EM is widely used in the fermentation process of organic matter and waste. It consists of photosynthetic bacteria, lactic acid bacteria, yeasts, and actinomycetes [6,7]. Photosynthetic bacteria form beneficial substances that produce amino acids, nucleic acids, and bioactive substances. Lactic acid bacteria function to ferment organic material into lactic acid, accelerate the decomposition of lignin and cellulose, and suppress pathogens in the presence of lactic acid produced. Yeasts such as Saccharomyces are decomposers of organic matter, reduce some types of poisons and anti-nutritional substances in food ingredients (such as phytates, tannins, and polyphenols) and increase the nutritional value of food ingredients [8-10]. Actinomycetes are gram-positive bacteria that are able to synthesize antibiotics and have extracellular enzyme activity which includes, nucleases, xylanases, chitinases, cellulases, lipases, and proteases [11-12] which are very important for hydrolyzing complex organic materials into simple components. Yeast produces antibiotic substances, enzymes, and hormones. Among the many types of EM, EM-4 is one type in the form of liquid probiotics containing Lactobacillus sp as much as $1.09 \times 10^7$ cfu/mL and Saccharomyces sp as much as $4.30 \times 10^7$ cfu/mL (based on the contents in the package, analysis results from the Laboratory of the Agriculture Faculty of Gadjah Mada University). Thus, EM-4 was suspected to has the potential to improve the quality of ingredients and feed by increasing the nutritional composition and digestibility of the ingredients and feed and eliminating or reducing anti-nutrient substances in the ingredients and feed. In this regard, this study was conducted to evaluate EM-4 as fermenter to feeds to improve feed quality and growth performance of rabbitfish, Siganus guttatus.

2. Materials and Methods

2.1. Test Diets

Five test diets were evaluated in this study, formulated with local ingredients including fish meal, soybean meal, copra cake meal, cornmeal, rice bran, seaweed sargassum meal (Table 1). Except for minerals and vitamins, all the ingredients were mixed homogeneously, then fermented with EM-4. The treatments were different doses of EM-4 as the fermenter at: 10 mL/kg (F10), 20 mL/kg (F20), 30 mL/kg (F30), 40 mL/kg (F40), and: diet without EM-4 fermenter (WF). The process of EM-4 fermentation was performed as follow:

(i) mixed 1 kg of the feed ingredients excluding vitamins and mineral components
(ii) diluted 50 mL molasses with 300 mL cold boiled water, then added EM-4 probiotics according to desired doses which were: 10 mL (F10), 20 mL (F20), 30 mL (F30), and 40 mL (F40)
(iii) mixed homogeneously with the prepared mixed ingredient of step (i).
(iv) incubated the mixture in a plastic bag placed in styrofoam for three days [13].
(v) added vitamins and minerals to the fermented ingredients
(vi) processed to the pellet diet.
2.2. Condition of Feeding Trial
The feeding trial was designed into a Completely Randomized Design consisting of five treatments with 3 replicates. It was carried out in The Floating Net Cage Installation of Research Institute for Coastal Aquaculture and Fisheries Extension, located in Aweraange Bay, Barru Regency, South Sulawesi Province. Cultured juvenile rabbitfish (second generation) with an average initial weight of 48.5±1.41g were used. Selected fish were randomly distributed into 15 units of 1 × 1 × 2 m³ net cages with a stocking density of 25 fish/cage. All diets were fed three times a day to apparent satiation (morning, noon, and afternoon). As soon as the fish had been satiated, feeding was stopped to minimize feed wastage. The fish were sampled every month during 120 days of culture.

| Ingredients          | Test diets |
|----------------------|------------|
|                      | WF | F10 | F20 | F30 | F40 |
| Fish meal            | 15 | 15  | 15  | 15  | 15  |
| Soybean meal         | 17 | 17  | 17  | 17  | 17  |
| Copra cake meal      | 20 | 20  | 20  | 20  | 20  |
| Corn meal            | 15 | 15  | 15  | 15  | 15  |
| Rice bran            | 15 | 15  | 15  | 15  | 15  |
| Sargassum meal       | 15 | 15  | 15  | 15  | 15  |
| Vitamin premix 1)    | 2  | 2   | 2   | 2   | 2   |
| Mineral premix 2)    | 1  | 1   | 1   | 1   | 1   |

Proximate composition:
- Protein: 25.1, 25.9, 26.3, 26.3, 25.8
- Lipid: 9.3, 9.0, 8.8, 8.5, 8.1
- Crude fibre: 9.8, 7.5, 11.0, 11.2, 11.4
- Ash: 12.2, 12.1, 12.0, 11.6, 11.9

1 Vitamin mix (in 1 kg of diet): Vit. A60,000 IU; Vit.D 20,000 IU; Vit.K 24 mg; Vit. E 150 mg; Vit B₁ 60 mg; Vit B₂ 90 mg; Vit B₆ 60 mg; Vit B₁₂ 60 mg; Vit C 160 mg; Calcium D-Pentathenate 80 mg; Folic acid 30 mg; Biotin 200 mg, Inositol 250 mg, Nicotiamide 400 mg, Cholin chloride 300 mg.
2 Mininal mix (in 1 kg of diet): Calcium 325 mg; Phosphor 100 mg; Iron 60 mg; Manganese 40 mg; Zinc 73.5; Copper 3 mg; Sodium 1 mg; Cobalt 1 mg; Iodine 0.75 mg; Potassium 0.035 mg.

2.3. Sample Collection and Chemical Analysis
Samples of fermented diets were analyzed for proximate, fatty acid, free-fatty acid, and amino acid. Fish samples obtained from initial and final experiment were analyzed for proximate carcass analysis. Proximate analysis was carried out according to [8] method. Moisture was analyzed after drying the samples at 105°C using the oven (Memmert, Germany) until constant weight, crude protein was determined by micro-Kjeldahl procedure, and lipid with gravimetrically determined by extracting petroleum benzene using Soxtherm apparatus, crude fiber by heating with alternating acid and base washing, and ash by combustion in a muffle furnace (Barnstead Thermolyne, CA, USA) at 550°C for 24 hours. Fatty acid profile was analyzed using Gas Chromatography (Shimadzu 2010 plus, Tokyo, Japan), free-fatty acid with titrimetry method, whereas amino acid analysis was carried out using high-performance liquid chromatography (HPLC) (Shimadzu 20A, Tokyo, Japan).

2.4. Calculation and Statistical Analysis
Biological responses of rabbitfish after 120 days feeding trial were evaluated for several parameters including:

Specific growth rate (SGR) [15]:

3
\[
SGR(\% \text{/day}) = 100 \times \frac{\ln W_e - \ln W_s}{d}
\]

Where \(\ln\) is the natural logarithm, \(W_e\) and \(W_s\) are the fish weights at the end and beginning of the study, respectively, and \(d\) is the day number for fish culture.

Feed intake \((F1, g \, DM \, fish^{-1}) = (total \, amount \, of \, dry \, feed \, consumed)/(fish \, numbers \times days)\)

Feed efficiency and protein efficiency ratio \([16]\).

\[
Feed \, efficiency = \frac{\text{Wet body weight gain (g)}}{\text{total dry feed consumed}}
\]

\[
Protein \, efficiency \, ratio, \, PER = \frac{\text{body weight gain (g)}}{\text{total feed consumed} \times \text{protein content in diets (g)}}
\]

\[
Survival \, rate, \, SR \, (\%) = \frac{\text{Final number of fish/Initial number of fish}}{\times 100\%}
\]

Data on the specific growth rate, survival rate, feed intake, feed efficiency, protein efficiency ratio, and body chemical composition were analyzed using a one-way analysis of variance (ANOVA). Significant differences \((P<0.05)\) were assessed using Tukey’s HSD test. All the statistical analyses were done using software SPSS version 21. Data on the content of free fatty acids, fatty acids, and amino acid of the test diets were analyzed descriptively.

### 3. Results and Discussion

The analysis results of the fatty acid and free fatty acid contents of the test diets are presented in Table 2. Some fatty acids are quite essential for common seawater commodities such as linoleic acid \((C18:2n-6)\), linolenic acid \((C18:3n-3)\), arachidonic acid \((C20:4n-6)\), eicosapentaenoic acid \((EPA, \, C20:5n-3)\), and docosahexaenoic acid \((DHA, \, C22:6n-3)\). Also, total n-3 and total n-6 were tended to have higher contents in a fermented diet with EM-4 at all doses than in diets without fermentation. However, the content of those fatty acids in fermented feed were relatively not much different for all EM-4 doses. The increased content of these fatty acids in fermented diets might be caused by the presence of bacteria or microbes in EM-4 that could convert these fatty acids. The EM-4 probiotic was used in this study contained microbes \(Lactobacillus\) sp and \(Saccharomyces\) sp. However, the ability of these bacteria and yeast causes in terms of increasing fatty acid content still needs to be studied further. Besides those kinds of fatty acids, the free fatty acid contents in all of the fermented diet with EM-4 were also higher than in an un-fermented diet, and the free fatty acid content tended to increase with the increasing use of EM-4 in the fermented diet. According to \([17]\), high free fatty acids content \((> 0.2\% \, of \, lipid)\) in food will cause an unpleasant flavor and sometimes can poison the body. The high content of free fatty acids in the fermented diet was due to the oxidation and hydrolysis process that is catalyzed by the lipase enzyme, which is likely to be produced by EM-4 microorganisms. The test diets were fermented with EM-4 for three days tend to undergo an oxidation process, which was characterized by the presence of a rancid odor when the fermentation containers were opened until the diets were ready to be pelleted. Even after drying the test diets, the rancid odor was reduced. This oxidation process was also triggered by a fairly high content of feed lipid (8-9%), which comes mostly from soybean meal and copra cake meal. In previous studies in fermentation using EM-4, the lipid content of the feed was only 6.5% \([13]\), and there was no sharp odor as sharp in this study.

### Table 2. Fatty acid profile (% lipid) and free fatty acid of fermented diet using EM-4 at different doses

| Fatty acid kinds | Test diets |
|-----------------|------------|
|                 | WF | F10 | F20 | F30 | F40 |
| C8:0            | 0.97 | 0.75 | 0.70 | 0.61 | 0.69 |
The amino acid content of the test diets is presented in Table 3. The table shows that the amino acid profile of the test diets was relatively the same for all treatments. This shows that the fermentation of the test diet with EM-4 has not improved the amino acid content of the test diets.

**Table 3.** Amino acid content (% dry matter) of test-diet fermented using EM-4 at different doses

| Kinds of amino acid | Test diets |
|---------------------|------------|
|                     | WF | F10 | F20 | F30 | F40 |
| **Non-essential:**  |    |     |     |     |     |
| Alanine             | 1.32| 1.37| 1.40| 1.24| 1.31|
| Aspartic acid       | 2.12| 2.21| 2.28| 2.02| 2.09|
| Glutamic acid       | 3.96| 4.10| 4.21| 3.69| 3.92|
| Glycine             | 1.19| 1.21| 1.24| 1.12| 1.17|
| Serine              | 0.92| 0.95| 0.99| 0.87| 0.90|
| Tyrosine            | 0.52| 0.56| 0.59| 0.52| 0.53|
| **Essential:**      |    |     |     |     |     |
| Arginine            | 2.20| 2.07| 1.78| 1.96| 1.62|
| Histidine           | 0.60| 0.61| 0.61| 0.57| 0.57|
| Isoleusine          | 1.01| 1.06| 1.07| 0.95| 1.01|
| Leusine             | 1.79| 1.90| 1.91| 1.69| 1.79|
| Lysine              | 1.39| 1.35| 1.40| 1.21| 1.22|
| Methionine          | 0.38| 0.40| 0.41| 0.32| 0.37|
Phenylalanine & 1.04 & 1.08 & 1.10 & 0.98 & 1.02 \\
Threonine & 0.88 & 0.90 & 0.92 & 0.82 & 0.86 \\
Valine & 1.25 & 1.27 & 1.29 & 1.15 & 1.22 \\
\hline
Total (%) & 20.57 & 21.05 & 21.18 & 19.11 & 19.59 \\
\hline

Growth performance and utilization of test diets during the study are presented in Table 4. The table shows that based on a statistical analysis of specific growth rates, survival rates, feed efficiency, and protein efficiency ratios, the results were not significantly different (P > 0.05) between treatments. While the highest feed intake was found in the fish fed un-fermented diets and significantly different (P < 0.05) with the fish fed the fermented diet for all treatments. Although statistically not significantly different, the fish fed un-fermented diet tended to have better growth performance, and feed utilization compared to the fish fed the fermented diets, this was caused by higher feed intake in the fish fed un-fermented diet compared to fish fed fermented diet. The difference in the feed intake was most likely due to the high content of free fatty acids in fermented diets compared to the un-fermented diet (Table 3). Free fatty acids are acids that are released in the process of hydrolysis and lipid oxidation by being catalyzed by the lipase enzyme. Free fatty acids, even though they are in small amounts, cause a bad taste. This occurs in lipid-containing non-volatile fatty acids, with the number of C atoms greater than 14 [18]. In addition, the fermented diet also has a fairly rancid odor when the fermentation container was opened until the diets were made pellet, even though the smell has decreased after the drying process. This causes the total amount of feed intake by the rabbitfish in fermented diets to be lower than in an un-fermented diet, and it has implications relatively lower of fish growth, and feed utilization for the fish fed fermented diets.

Table 4. Growth performance of the fish test and utilization rate of test diets

| Variables                        | WF     | F10    | F20    | F30    | F40    |
|----------------------------------|--------|--------|--------|--------|--------|
| Initial body weight (g/ind)      | 48.8±0.8 | 48.0±1.1 | 48.7±1.4 | 48.1±1.8 | 48.9±2.0 |
| Final body weight (g/ind)        | 165.8±6.3 | 150.6±9.6 | 155.0±3.4 | 150.0±0.5 | 153.9±9.4 |
| Specific growth rate (%/day)     | 1.02±0.03a | 0.95±0.05a | 0.97±0.01a | 0.95±0.03a | 0.95±0.04a |
| Survival rate (%)                | 100    | 100    | 100    | 100    | 100    |
| Feed intake (g/ind.)             | 295.1±3.6b | 265.6±3.9b | 274.6±4.2b | 268.2±1.9b | 271.1±6.8b |
| Feed efficiency (%)              | 39.6±1.7a | 38.6±2.8a | 38.7±0.1a | 38.0±0.9a | 38.7±3.0a |
| Protein efficiency ratio         | 1.58±0.07a | 1.49±0.11a | 1.47±0.01a | 1.44±0.03a | 1.50±0.12a |

Value in each row with different superscripts have significant differences (P<0.05)

Another interesting thing from this research is the content of polyunsaturated fatty acids (PUFA) such as arachidonic acid, EPA and DHA are higher in all diets that were fermented with EM-4 compared to un-fermented diet, but the growth performance of rabbitfish was not significantly different in among all treatments, including those that were fed without fermenting. Whereas arachidonic acid, EPA and DHA are known to be essential fatty acids for most marine fish which are very important for the structure of components in cell membranes [19], growth and reproduction [20, 21], eicosanoid precursors [22], osmoregulation [23], regulators of gene expression [24]. The growth was also still high in the fish fed an un-fermented diet (containing lower PUFA fatty acids) due to rabbitfish, Siganus guttatus, this also may have the ability to synthesize these fatty acids, especially polyunsaturated fatty acids. The results of the study reported by [25] and [26] concluded that rabbitfish, Siganus canaliculatus, could convert linoleic and linolenic fatty acids into PUFA, where the conversion capacity is higher at low salinity.
compared to high salinity. Other marine fish that are able to synthesize PUFA include Atlantic salmon (Salmon salar) [27] and red sea bream (Pagrus major) [28]. The existence of this fish's ability to synthesize PUFA will provide opportunities for the use of fish oil in the rabbitfish diet, which can be minimized or even eliminated.

### Table 5. Proximate composition of the whole body in rabbitfish fed different test diets (% dry matter)

| Body composition | Test diets |
|------------------|------------|
|                  | WF         | F10  | F20   | F30   | F40   |
| Protein          | 59.1±2.7   | 61.3±1.7  | 60.5±0.4 | 60.6±0.9 | 59.3±0.5 |
| Lipid            | 24.1±1.1   | 24.3±1.4  | 24.1±1.3  | 23.7±0.8  | 25.7±1.6 |
| Crude fibre      | 3.1±0.6    | 1.7±1.9   | 2.4±1.4   | 4.4±0.5   | 2.8±1.1  |
| Ash              | 11.5±0.7   | 11.5±1.1  | 12.1±1.5  | 11.1±0.6  | 11.7±0.1  |

The proximate composition of whole-body fish at the end of the study is presented in Table 5. The table shows that the protein, lipid, crude fiber and ash content of the fish were not significantly different (P> 0.05) between treatments. This shows that the test diets applied did not have an effect that could change the proximate composition of the whole body of the rabbitfish, although the fermented diets tended to be consumed less than the un-fermented diet by rabbitfish.

### 4. Conclusion
- Feed fermented with EM-4 at all doses tended to increase the content of arachidonic acid (ARA), eicosapentanoic acid (EPA), and docosahexaenoic acid (DHA), total n-3, total n-6, and free-fatty acid.
- Specific growth rate, weight gain, feed efficiency, and the protein efficiency ratio was not significantly different between treatments; however, feed intake lower in fish fed the fermented diet compared to fish fed the un-fermented diet.
- The use of EM-4 in several doses in feed fermentation has not been able to increase feed utilization and growth performance of rabbitfish.

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