HAYATI
Journal of Biosciences

Vol. 30 No. 1, January 2023 140-147
DOI:10.4308/hjb.30.1.140-147
ISSN: 1978-3019
EISSN: 2086-4094

Effects of Dietary Mannan-Oligosaccharide (Mos) and Multi-Species of *Bacillus* on Growth and Feed Utilization in Leopard Coral Grouper *Plectropomus leopardus* Juvenile

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ARTICLE INFO

Article history:
Received October 12, 2021
Received in revised form July 5, 2022
Accepted August 8, 2022

KEYWORDS:
leopard coral grouper,
mannan-oligosaccharide,
multi-species of *Bacillus*,
Plectropomus leopardus

ABSTRACT

This study assessed the growth and feed utilization in leopard coral grouper *Plectropomus leopardus* juvenile fed dietary prebiotic mannan-oligosaccharide (MOS) and multi-species of probiotic bacteria (*Bacillus cereus* BS6, *B. subtilis* BS3, and *B. amiloliquefaciens* BS4). The experiment was performed by feeding four replicates groups of juveniles weighing 3.61±0.60 g on four experimental diets, and each diet included: only MOS (PRE); the multi-species of *Bacillus* (PRO); a combination of MOS and the multi-species of *Bacillus* (SYN) and without supplementation (CON). When MOS (PRE) or the multi-species of *Bacillus* (PRO) was included in the diet solely, the growth of the fish was fairly good, although there was no significant difference with the control diet ($p>0.05$). Unexpectedly, the combined inclusion of MOS and the multi-species of *Bacillus* (SYN) did not increase the growth and feed utilization in *Plectropomus leopardus* juvenile. Presumably, MOS did not exert any favorable effects on the multi-species of *Bacillus*. This study suggested that no advantage was obtained when MOS and the multi-species of *Bacillus* were included in the diet simultaneously. In addition, the inclusion of MOS in higher levels may be necessary for better feed utilization and growth in leopard coral grouper *Plectropomus leopardus*.

1. Introduction

Leopard coral grouper (*Plectropomus leopardus*), locally known as "kerapu sunu" (in Indonesia), is distributed along the Indo-Pacific regions from Indonesia to Fiji and from Japan to Australia. The wholesale price of live *Plectropomus leopardus* averaged US$ 78.8/kg, much higher than the wholesale price of hybrid groupers that only US$ 14.1/kg (Sadovy *et al.* 2017). Indonesia has been the major supplier of *Plectropomus leopardus* since the mid-2000s. Some countries had claimed to cultivate this species through hatchery-based aquaculture. However, the commercial culture was unclear (Sadovy *et al.* 2017). Institute for Mariculture Research and Fisheries Extension (IMRAFE) in Gondol-Bali, Indonesia, has been developing *Plectropomus leopardus* hatchery technology, and mass production of fingerlings has been accomplished with a survival rate achieved to 11.8% (Giri *et al.* 2020). Even though *Plectropomus leopardus* was a highly desirable grouper fish, this species' growth was low, as Setiawati *et al.* (2017) reported.

Generally, fish growth can be improved by utilizing dietary prebiotics and probiotics. Among the prebiotics and probiotics frequently used in aquaculture are mannan-oligosaccharide (MOS), fructooligosaccharide (FOS), and *Bacillus* sp. Dietary prebiotic MOS had been examined in the nursery of *Plectropomus leopardus* fingerlings; however, further studies are needed to determine levels of MOS inclusions to obtain optimal growth (Marzuqi *et al.* 2021). There had been confirmed that dietary prebiotics and probiotics improved the growth of cultured fish (Afrilasari *et al.* 2016; Jayaprakash and Parvathi 2019). However, several studies did not find the positive effects of prebiotics and probiotics on fish growth, as Sado *et al.* (2014) stated. Hence, regarding the significant variations and even contradictive results reported in the literature, the

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use of prebiotics and probiotics in fish needs to be investigated based on species.

Astuti (2018) successfully isolated potentially probiotic bacteria from the wild *Plectropomus leopardus* digestive tract, identified as *Bacillus cereus* BS6, *B. subtilis* BS3, and *B. amyloliquefaciens* BS4. These probiotic bacteria demonstrated proteolytic and amylolytic activities and improved the growth of *Plectropomus leopardus* juvenile. However, those probiotic bacteria were administered to the diet as a single species and without prebiotics supplementation. Therefore, it is clear that the use of prebiotics and multi-species of probiotics has not been examined in *Plectropomus leopardus*. In the present study, an experiment was done to evaluate the effects of dietary prebiotic mannan-oligosaccharide (MOS) and the three species of probiotic bacteria, i.e., *Bacillus cereus* BS6, *B. amyloliquefaciens* BS4, and *B. subtilis* BS3 on feed utilization and growth of *Plectropomus leopardus* juvenile.

2. Materials and Methods

2.1. Preparation of Probiotic Bacteria

This study used the multi-species of *Bacillus* isolated from the gut of wild *Plectropomus leopardus*, which was maintained in 15% glycerol at –20°C by Astuti (2018). The probiotic bacteria *Bacillus cereus* BS6, *B. subtilis* BS3, and *B. amyloliquefaciens* BS4 were cultivated in Sea Water Complete (SWC) media and incubated for 24 hours. The growing bacterial colonies were then subcultured in the SWC broth media, 10 ml in volume, for 20 hours. The probiotic bacteria were re-cultured in a higher volume of SWC broth media at 250-500 ml for 20 hours. Lastly, the bacteria were harvested and used for diet preparation.

2.2. Preparation of the Experimental Diets

The experimental diets were formulated to contain 0.6% MOS (PRE) and 1% probiotics which consisted of the same amount of *Bacillus cereus* BS6, *B. subtilis* BS3, and *B. amiloliquefaciens* BS4 (PRO), 0.6% MOS + 1% of the multi-species of *Bacillus* (SYN), and the control diet (CON) (Table 1). The ingredients of each diet (Table 1) were thoroughly mixed. About 25-30% water was added to the mixture. The mixture was then extruded through a pelletizing machine (Hiraga, Japan) to produce a 2-mm-diameter pellet. The pellets were air-dried for 3 hours, packed in plastic bags, and stored at -20°C for the feeding experiment.

2.3. Experimental Design and Feeding Trial

Plectropomus leopardus juveniles were harvested from the hatchery of IMRAFE in 2019. Then, the juveniles were transferred to the wet Lab. of Feed and Nutrition. The fish were acclimatized to the laboratory conditions in a fiberglass tank (2 x 2 x 1 m^3) equipped with aeration. The acclimatization process was done in a flow-through water system

Table 1. The experimental diets ingredients (%) and proximate composition (%DM)

Ingredients	Prebiotic (PRE)	Probiotic (PRO)	Synbiotic (SYN)	Control (CON)
Fish meal	50.10	50.10	50.10	50.10
Squid liver meal	12.00	12.00	12.00	12.00
Mysid meal	10.00	10.00	10.00	10.00
Soybean meal	10.00	10.00	10.00	10.00
Wheat flour	9.82	9.42	8.82	10.42
Fish oil	2.48	2.48	2.48	2.48
Vitamin mix	1.30	1.30	1.30	1.30
Mineral mix	1.70	1.70	1.70	1.70
Taurin	0.50	0.50	0.50	0.50
MOS	0.60	0.00	0.60	0.00
Multi-species of *Bacillus*	0.00	1.00	1.00	0.00
Binder (CMC)	1.50	1.50	1.50	1.50
Total	100.00	100.00	100.00	100.00
Proximate composition				
Protein	47.05	50.45	47.67	47.08
Lipid	12.74	11.80	12.07	12.15
Ash	19.48	19.79	19.90	19.17
Fiber	4.52	3.89	3.74	3.74
NFE	16.21	14.08	16.62	17.86

NFE: nitrogen-free extract, calculated as 100 - (protein + lipid + ash + fibre)

at a range temperature of 29.10–30.20°C, dissolved oxygen (DO) 5.02–5.92 mg/L, and salinity 31–32 ppt. These water quality parameters were suitable for *Plectropomus leopardus* culture (Setiawati *et al.* 2017). The fish were fed the control diet to apparent satiation for one week before the start of the experiment. Hereafter, the juveniles (average weight 3.61±0.60 g) were randomly allocated into 16 fiberglass tanks, 300 L in volume, at 50 fish per tank. A Completely Randomised Design (CRD) with four diet treatments as prepared was established in this study. Four replicate tanks for each diet treatment were set up. A flow-through water system (6 L/min.) with aeration was applied in the tanks. The juveniles were fed the experimental diets until satiated three times/day; at 08:00 am, 1:00 pm, and 3:30 pm. A feeding trial was performed for 12 weeks. The apparent digestibility coefficient (ADC) of dry matter, protein, and lipid was assessed. This study also measured weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein productive value (PPV) and lipid productive value (LPV), as well as survival rate (SR).

2.4. Proximate Analysis

Proximate analyses were carried out in the IMRAFE's Chemical Lab. The test diets and feces were analyzed to determine digestibility, whereas proximate analysis of the fish's whole body was used to determine protein and lipid productive values. Proximate analyses were done based on standard methods (AOAC 2005).

2.5. Digestive Enzyme Analysis

The fish were starved to empty their digestive tract from 09.00 am until the next 24 hours after the feeding trial ended. Two fish were selected at random from each tank. The intestinal tract of the fish samples was removed. The intestine was then washed tenderly with aquadest, weighed, and transferred into a-1.5 ml sterile tube, and homogenized in pH 7.5 Tris buffer (20 mM Tris HCl, 1 mM EDTA, 10 mM CaCl2) (0.1 g intestinal tract/0.9 ml buffer). Centrifugation of the homogenate was done at 10,000 rpm for 20 min at 4°C (Wang *et al.* 2015). The obtained supernatants (the enzyme extract of the intestine) were transferred into new tubes and stored at 4°C before digestive enzyme analysis. Analysis of digestive enzymes was done by the spectrophotometer method. Lipase activity was analysed by the method of Tietz and Friedrick in Borlongan (1990). Amylase and protease were determined according to Bergmeyer and Grassi (1983) in Natalia *et al.* (2004), and cellulase was analysed by the method of Ghose (1987).

2.6. Histological Examination of the Intestine

Two fish from each treatment were randomly sampled for histological analysis at the termination of the experiment. The intestine was removed and fixed in Bouin's solution for 6 hours after dissecting the fish. Dehydration of the sample intestine was done in a serial alcohol solution of 70%, 80%, 90%, 95%, and 100%, and it was then cleared in xylene. Subsequently, the samples were embedded in paraffin. The embedded tissues were dissected into 3-5 µm thickness, mounted on glass slides, allowed to dry overnight, and stained with Hematoxylin and Eosin (H and E). Each section was examined under a light microscope (Nikon ECLIPSE E600) at 400x magnification (Mahardika *et al.* 2012) which was connected to a camera (Nikon Digital Camera DXM1200F). An image capture system (ACT program) was used to convert all images of each section into a digital form.

2.7. Intestinal Bacteria Populations

Intestinal bacteria populations were assessed according to the method of Astuti (2018). At the end of the feeding trial, the fish were starved for 24 hours, and four fish from each treatment were randomly sampled. The fish samples were dissected to remove their intestines. The number of probiotic bacteria was estimated using the heat-shock method. The intestines were heated at 80°C for 15-20 minutes. This temperature was applied considering that spores of *Bacillus cereus* and *B. subtilis* survived at the temperature of 95–120°C (Janstova and Lukasova 2001). At the same time, the intestine samples for total bacteria (TB) count were unheated. One gram of each intestine sample was transferred into a sterile tube and homogenized in 9 ml sterile phosphate-buffered saline (PBS). Phosphate-buffered saline was also used to serially dilute the suspension from 10^{-1} to 10^{-3}. Subsequently, its 100 µl portions were spread on duplicate plates of SWC media. All plates of bacteria were incubated at room temperature. Bacterial colonies were counted after 24 hours. Before statistical analysis, data of bacteria counts were first presented as CFU/g and subsequently transformed into log10 values.

2.8. Statistical Analysis

A one-way analysis of variance (ANOVA) by the Rcommander program was used to analyse the data. When ANOVA confirmed the significant effects of treatments on the experimental parameters, Duncan Multiple Range Test (DMRT) was carried out to examine significant differences among treatments. Statistically significant differences in means among treatments were accepted at $p<0.05$ (Fox 2005).

3. Results

3.1. Digestibility Analysis

Feeding with dietary prebiotics and probiotics affected the apparent digestibility coefficient (ADC) in leopard coral grouper juvenile. It is seen that a single application or combination of MOS and the multi-species of *Bacillus* significantly affected the nutrient digestibility ($p<0.05$) (Table 2). Dietary MOS combined with the multi-species of *Bacillus* (SYN) showed the highest ADC of dry matter and protein, 67.78±0.73% and 89.21±0.02%, respectively. Lipid ADC in fish fed synbiotic was also high, followed by prebiotic and probiotic groups. Fish fed the control diet exhibited low digestibility of protein and lipid.

3.2. Growth Performance, Feed Utilization, and Survival

Individual application of dietary prebiotic or probiotic increased the growth of leopard coral grouper juvenile compared to the combined inclusion of prebiotic and probiotic (SYN). Dietary prebiotics or probiotic produced a reasonably good growth, with a final weight of 15.62±1.91 g and 15.36±1.55 g, respectively. These results were significantly different from the synbiotic treatment, with a final weight of only 14.14±1.62 g ($p<0.05$), but these were not significantly different from the control, which was 14.72±0.66 g ($p>0.05$) (Table 3). Similarly, the weight gain and the specific growth rate in the PRE and PRO treatments were relatively high, although not significantly different from the control treatment. This growth performance was related to feed consumption and feed conversion ratio, which was similar among treatments. In addition, the PRE and PRO treatments resulted in protein and lipid productive values similar to the control treatment ($p>0.05$). Survival was also the same among treatments.

3.3. Intestinal Bacteria Populations

Counts of the presumptive probiotic bacteria (PBB) and total bacteria (TB) show varied results among treatments (Table 4). These results indicated that feed supplementation with synbiotics or probiotics increased the TB in the intestine, other than the PBB. The total bacteria in the SYN was higher than in the PRE and the CON ($p<0.05$) but not different from the PRO treatment ($p>0.05$).

3.4. Digestive Enzyme Activities

Generally, all the treatments resulted in the same activities of cellulase, amylose, and lipase ($p>0.05$). However, protease was higher in the probiotic than in the control treatment ($p<0.05$). The level of protease was the same between prebiotic and synbiotic and not significantly different from the control treatment

Table 2. Apparent digestibility coefficient (ADC) of dry matter, protein, and lipid in juvenile *Plectropomus leopardus* fed dietary prebiotics and probiotics

	Prebiotic (PRE)	Probiotic (PRO)	Synbiotic (SYN)	Control (CON)
Dry matter ADC (%)	63.30±1.29^{b}	59.49±0.60^{a}	67.78±0.73^{c}	61.44±0.89^{ab}
Protein ADC (%)	87.63±0.10^{b}	87.19±0.04^{b}	89.21±0.02^{c}	86.43±0.05^{a}
Lipid ADC (%)	78.02±0.77^{ab}	77.36±1.12^{ab}	78.87±2.67^{b}	74.68±0.41^{a}

Table 3. Growth, feed utilization, and survival of juvenile *Plectropomus leopardus* fed the experimental diets for 12 weeks

Parameters	Prebiotic (PRE)	Probiotic (PRO)	Synbiotic (SYN)	Control (CON)
Final weight (g)	15.62±1.91^{b}	15.36±1.55^{b}	14.14±1.62^{a}	14.72±0.66^{ab}
Weight gain (%)	345.39±18.18^{b}	338.01±15.31^{b}	273.16±29.08^{a}	306.62±20.33^{ab}
Specific growth rate (%/day)	1.74±0.12^{b}	1.71±0.09^{b}	1.61±0.10^{a}	1.66±0.05^{ab}
Feed consumption (g/fish)	18.07±0.91^{a}	19,49±0,82^{a}	18.86±0.91^{a}	18.28±1.29^{a}
Feed conversion ratio	1.63±0.11^{a}	1.67±0.18^{a}	1.81±0.21^{a}	1.64±0.07^{a}
Protein productive value	18.75±1.77^{a}	17.73±0.71^{a}	16.35±2.12^{a}	17.32±3.28^{a}
Lipid productive value	7.15±1.07^{a}	6.93±2.08^{a}	6.21±0.51^{a}	6.26±2.07^{a}
Survival (%)	96.66±2.30^{a}	97.50±1.00^{a}	96.00±1.63^{a}	99.50±1.00^{a}

Data are presented as mean ± SD, derived from four replicate tanks (n = 4)
Different superscript letters in the same row indicate a significant difference between treatments ($p<0.05$)

(Table 5). A high level of protease in the probiotic treatment indicated that the multi-species of *Bacillus* were able to produce extracellular enzymes.

3.5. Histological Examinations of the Intestine

Histological examination of the intestine revealed that dietary MOS and the multi-species of *Bacillus* increased the surface area of the villous intestine, indicated by the helical structure of the villi. It was likely that a higher surface area of the villi increased nutrient absorption in the intestine. On the other hand, the villi structure of the control fish was in straight structure, and, consequently, its surface area was the lowest compared to those of the other treatments (Figure 1).

The histological examination showed that juvenile *Plectropomus leopardus* fed dietary prebiotic (PRE), probiotic (PRO), and synbiotic (SYN) had higher villous fold length than juveniles fed the control diet (CON) (Figure 1). Moreover, an increase in the epithelial thickness of the intestine was seen in juveniles fed dietary prebiotic, probiotic, and synbiotic.

Table 4. The presumptive probiotic bacteria (PBB) and total bacteria (TB) populations (Log CFU/g) in the intestine of juvenile *Plectropomus leopardus*

	Presumptive probiotic bacteria (PB)	Total bacteria (TB)
Prebiotic (PRE)	3.36±0.33[a]	5.79±0.10[a]
Probiotic (PRO)	4.15±0.31[b]	6.06±0.07[ab]
Synbiotic (SYN)	3.90±0.59[ab]	6.48±0.70[b]
Control (CON)	3.70±0.07[ab]	5.77±0.25[a]

4. Discussion

4.1. Digestibility Analysis

This study indicated that dietary prebiotics and probiotics resulted in a high ADC of dry matter, protein, and lipid. As found in other studies, dietary prebiotics and probiotics commonly improved the digestibility of nutrients in fish. A previous study on the single inclusion of *Bacillus subtilis, B. cereus*, or *B. amyloliquefaciens* for leopard coral grouper juvenile

Table 5. Digestive enzyme activities of juvenile *Plectropomus leopardus* fed the experimental diets for 12 weeks

	Prebiotic (PRE)	Probiotic (PRO)	Synbiotic (SYN)	Control (CON)
Cellulase (unit/ml)	0.08±0.01[a]	0.10±0.04[a]	0.09±0.02[a]	0.10±0.03[a]
Amylase (IU/ml)	0.98±0.14[a]	0.98±0.23[a]	0.89±0.20[a]	0.96±0.23[a]
Lipase (IU/ml)	0.07±0.01[a]	0.07±0.00[a]	0.06±0.01[a]	0.07±0.01[a]
Protease (IU/ml)	0.56±0.18[ab]	0.67±0.21[b]	0.53±0.15[ab]	0.43±0.08[a]

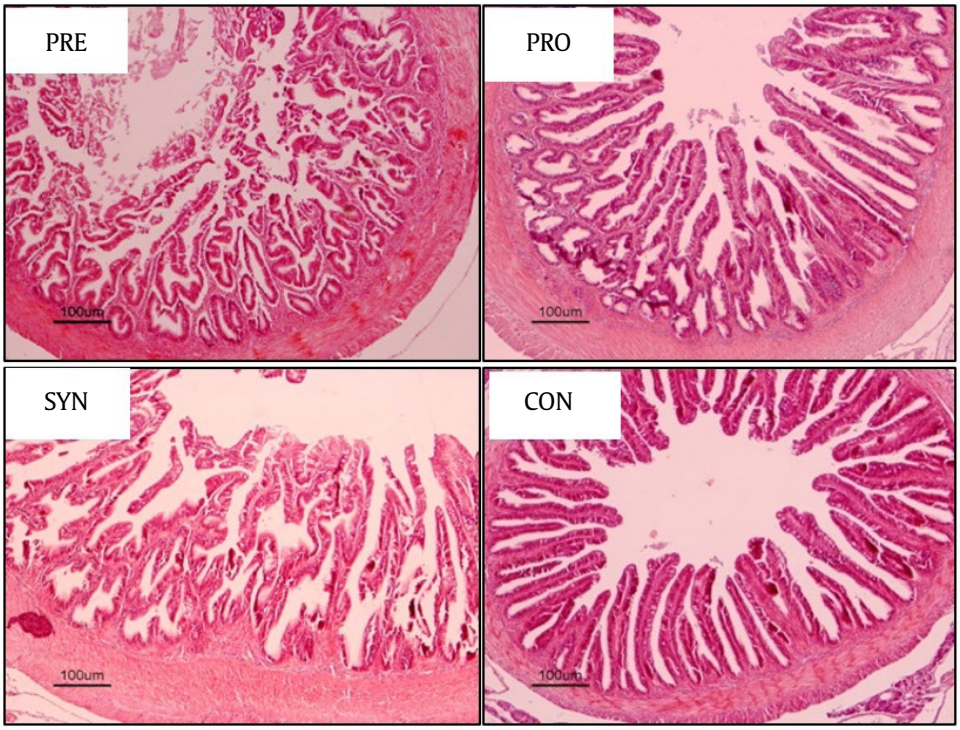

Figure 1. Intestinal villi structure of juvenile *Plectropomus leopardus* fed dietary prebiotic (PRE), probiotic (PRO), synbiotic (SYN), and the control diet (CON) for 12 weeks

found significantly high protein digestibility (Astuti 2018). Increased total nutrient digestibility and protein digestibility in humpback grouper-fed prebiotics and probiotics had been reported as well by Marlida *et al.* (2014). However, no significant effect of dietary probiotics on the apparent digestibility of lipid in rohu, *Labeo rohita* (Mohapatra *et al.* 2012).

The improved digestibility of nutrients might be correlated with the increased digestive enzymes synthesized by probiotics which allow the nutrients to be degraded more by the host (Mohapatra *et al.* 2012). The present study found that the combined MOS and the multi-species of *Bacillus* had the highest protein ADC. However, its productive protein value was similar to the other treatments (Table 3). In this case, it was likely that the utilization efficiencies of digestible protein were either steady or prone to plateau with the increase in protein intake (Pirozzi *et al.* 2010).

4.2. Growth Performance, Feed Utilization, and Survival

This study found that the growth of the juvenile improved slightly with a single inclusion of prebiotic or probiotic. However, the growth rate was hampered when the prebiotic and probiotic were included simultaneously. This is the most important finding in this study that will be discussed further.

Several researches indicated that MOS has advantageous effects on cultured fish's growth. But, the present study found that MOS gave only a slightly higher growth than the control diet. This study's findings were in line with several studies which found that MOS did not affect fish growth, such as in sturgeon fish (Pryor *et al.* 2003). The gut microbiota of the fish probably does not respond to MOS additives. Another consideration is that it may be necessary to include MOS in higher proportions for better responses of gut microbiota and growth of the fish (Pryor *et al.* 2003).

Although not many references are available concerning the effect of multi-species of probiotic bacteria on growth to compare with our findings, our results contradict a previous study which proved that the inclusion of single species of *Bacillus subtilis* BS3, *B. amyloliquefaciens* BS4 or *B. cereus* BS6 as probiotics improved growth, feed utilization, digestibility of total nutrient, protein, carbohydrate, and lipid, as well as retention of protein and lipid in leopard coral grouper (Astuti 2018).

The present study found that the combined inclusion of MOS and the multi-species of *Bacillus* (SYN) did not improve the growth of the fish. This study contradicts a hypothesis that probiotic bacteria would be more effective in increasing fish growth when prebiotics are included in the feed ingredients. The discrepancy of the results with the assumption was suspected because the prebiotic (MOS) was unable to stimulate the growth of the three species of probiotic bacteria, *Bacillus subtilis* BS3, *B. amyloliquefaciens* BS4, and *B. cereus* BS6. These results were supported by data on the counts of the presumptive probiotic bacteria in the synbiotic treatment (SYN) that were not different from those of the probiotic treatment (PRO) (Table 4).

A single inclusion or combined administration of prebiotics and probiotics inconsistently influences cultured fish's growth performance (Ye *et al.* 2011). A study on large yellow croaker *Larimichthys crocea* showed that at each prebiotic inclusion level, the supplementation of *Bacillus subtilis* improved the specific growth rate (SGR). However, at each *B. subtilis* inclusion level, the supplementation of prebiotics did not significantly improve the SGR and survival rate (Ai *et al.* 2011). Thus, it was suggested that future research should consider that only particular combinations of prebiotics and probiotic which provide synergistic results could be defined as synbiotics (Lin *et al.* 2012).

Juvenile leopard coral grouper at the end of the experiment had survival rates ranging from 96.0-99.5%. These survival levels did not differ among treatments, which indicated that the feeds with the inclusion of MOS and the three species of *Bacillus* did not negatively affect the survival rate of the fish. Mortality in this study occurred because of competition in foraging food. Fish did not grow and finally died. The survival rate obtained in this study was higher than in the previous research, which was only 60-70% (Astuti 2018). Survival in this study was comparable to those of the studies on prebiotic and probiotic supplementation in feed for juvenile humpback grouper, which resulted in a 100% survival rate (Marlida *et al.* 2014).

4.3. Intestinal Bacteria Populations

In this study, the presumptive probiotic bacteria were cultivated from the fish intestines that were previously heated at 80°C for 15-20 minutes. Therefore, the colonies of bacteria resistant to a temperature of 80°C, which were also found in the PRE and the CON groups, possibly originated from the microflora normal of the fish since the diets in these groups were not supplemented with the probiotic bacteria. The PBB populations obtained in this study were lower than in the previous study by Astuti (2018). The PBB populations were around 3.36-4.15 Log CFU/g, much lower than the results

of the study by Astuti (2018), which reached 6.12-6.49 Log CFU/g. Finding in this study indicated that the growth capability of the probiotic bacteria was lower or had decreased since it was discovered by Astuti (2018).

The count of the presumptive probiotic bacteria (Table 4) indicated that although the diet in the SYN was added with the combination of MOS and the three species of *Bacillus*, MOS did not promote the growth and colonization of the probiotic bacteria. Seemingly, the probiotic bacteria were unable to utilize MOS optimally or were not capable of metabolizing the available mannan-oligosaccharide, which resulted in the lower PBB populations (Table 4). Another possibility was that the proportion of MOS in the diets may need to be increased to promote the growth of the probiotic bacteria.

Synbiotic effects depend on several factors, such as substrate preferences of probiotic bacteria (Rastall and Maitin 2002), as well as the environment (Ai *et al.* 2011). Most importantly, it depends on species of fish, levels of inclusion, length of feeding, and the synergetic actions between prebiotics and probiotics (Cerezuela *et al.* 2011). Therefore, the present study suggested that it is necessary to examine further the effects of prebiotic MOS on the growth of each of the three *Bacillus* species by in vitro study before the *in vivo* study. This agrees with the statement of Cerezuela *et al.* (2012), which stated that *in vitro* assessment of the effects of any prebiotics on the selected probiotic is crucial to confirm whether they enhance or inhibit the growth of the selected probiotic.

4.4. Digestive Enzyme Activities

Data on digestive enzyme activities (Table 5) indicate that dietary multi-species of probiotic bacteria (PRO) increased protease activity. This increase was probably related to the high count of the presumptive probiotic bacteria (PBB) in the PRO group (Table 4). Animals could produce high digestive enzyme activities due to the presence of probiotic bacteria, which promoted the synthesis of endogenous digestive enzymes (Mohapatra *et al.* 2012). Protease activity in the PRO group was higher than in the CON group; however, its growth and productive protein value were similar to the control group (Table 3). Protein was probably mainly used as an energy source which caused low protein productive value (Tran-Ngoc *et al.* 2019). An earlier study declared that although the increased protease activities in fish promoted weight gain, digestive enzyme activity did not exhaustively represent feed efficiency (Ye *et al.* 2011).

4.5. Histological Examinations of the Intestine

Dietary MOS and the multi-species of *Bacillus* increased the surface area of the intestinal villi. Consistent with the present study, yellow tail lambari *Astyanax bimaculatus* fed dietary probiotic *Lactobacillus* spp. showed higher width, length, and perimeter of intestinal villi than fish fed the control diet (Jatoba *et al.* 2017). Higher length and perimeter of intestinal villi indicated higher surface area, which promotes better absorption of the available nutrients (Jatoba *et al.* 2017), which could elucidate a modest improvement in the growth performance of fish-fed dietary probiotics (Gisbert *et al.* 2013). However, a study on feed supplementation with prebiotic (MOS) in Gulf sturgeon did not show any effects on the gastrointestinal morphology (gastric length) or the spiral structure of the villi (length, width, and density of villi) of the fish (Pryor *et al.* 2003). In general, the apparent differences in the effects of dietary MOS on gastrointestinal tract morphological structure appeared to be species-specific (Anguiano *et al.* 2013).

This study concluded that single supplementation of MOS or the multi-species of *Bacillus* in diets resulted in relatively good growth. However, dietary MOS combined with the multi-species of *Bacillus* could not improve feed utilization, digestive enzymes, and growth in leopard coral grouper juvenile.

Conflict of Interest

The authors hereby declare that there is no conflict of interest.

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