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Effect of the Supplementation of Endogenous Probiotics in Feed on Growth Performance, Digestive Enzyme Activity, and Non-Specific Immunity Gene Expression of Sandfish, *Holothuria scabra* Juveniles

Sari Budi Moria Sembiring[1,2*], Jhon Harianto Hutapea[2], Ketut Mahardika[2], I Nyoman Adiasmara Giri[2], Rarastoeti Pratiwi[1], Haryanti[2], Suwarno Hadisusanto[2]

[1]*Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia*
[2]*Research Center for Fishery, Research Organization for Earth Sciences and Maritime, National Research and Innovation Agency, Bogor 16912, Indonesia*

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ABSTRACT

The present study aimed to evaluate the effects of endogenous compared to commercial probiotics on the growth, digestive enzyme activity, and non-specific immunity of sandfish juveniles. Treatments were: A. diet without probiotic; B. diet supplemented with endogenous probiotics *Gamma proteobacterium* strain M-4, *Bacillus subtilis* strain Q-1, *Bacillus* sp. strain E-2; C. diet supplemented with a commercial probiotic containing *B. subtilis*, *B. licheniformis*, and *B. pumilus*. The research was conducted for 4 months using 12 plastic containers, 30 juveniles each (initial weight of 2.72±0.37 g, mean ± standard deviation). From each container, ten samples were taken randomly every 14 days and weighed and three intestine samples at initial and every following month to analysis enzyme activities. At the end the study, all juveniles were counted and weighed individually, and performed a challenge test by injected a 10^6 CFU ml^{-1} *Vibrio azureus* strain 4C-1 at 0.1 ml/ind. Immune response observed were: total coelomate and gene expression (SOD, CAT, LZM) by Quantitative real-time PCR. The specific growth rate of juveniles fed by diet B was significantly different from treatment A ($P<0.05$), its final weight was higher compared to diets A and C ($P<0.05$). Protease, lipase, and amylase activity in treatment B was higher compared to C and A ($P<0.05$). The survival rate, total coelomocyte count, gene expression after being challenged were higher in treatment B compared to C and A ($P<0.05$). The present study showed endogenous probiotics promote growth, improve digestion enzyme activity, and stimulate non-specific immune responses.

1. Introduction

Since the 1980s, sandfish (*Holothuria scabra*) has become a marine fishery commodity with high economic value in the food industry, especially in the Asian market (Junus *et al.* 2018). Indonesia is one of the leading suppliers of sea cucumber products, but almost all of them are natural products. Cultivation of sandfish has to promote using juveniles from hatcheries to anticipate the decline of wild stock due to overfishing. Seedlings of sandfish in Indonesia have been successful, but the growth rate and survival of juveniles in the nursery tanks are still low (30-40%). Several factors cause juvenile sandfish death, such as changes in environmental conditions (temperature and salinity) and *Vibrio* sp. infection, which spreads to other individuals (Delroisse *et al.* 2020; Kunhold *et al.* 2019; Tangestani and Kunzman 2019).

Administration of vaccines (Mondal and Thomas 2022), immunostimulants (Khanjani *et al.* 2022), and probiotics (Simon *et al.* 2021) have been reported to increase the immunity and resistance of aquaculture animals to disease. Probiotics, defined as micro-organisms or their products that benefit the host's health (FAO/WHO 2001), were known for their antagonism to the pathogen, enhancement of immune response and feed efficiency, improvement of micro-flora balance in animals (Anee *et al.* 2021).

Studies on the application of probiotics in sandfish have been carried out by (Abdelaty *et al.* 2021;

* Corresponding Author
E-mail Address: moriasembiring@gmail.com

Sembiring *et al.* 2020) and not so many if compared to *A. japonicus* (Li *et al.* 2015; Wang *et al.* 2015; Yan *et al.* 2014; Yang *et al.* 2015; Zhao *et al.* 2016). They reported that probiotic supplemented in diet was effectively to promote growth, increase immunity against pathogenic bacterial and virus infections.

Three bacterial isolates from the intestines of natural sandfish were obtained as probiotics, and three isolates were *Gamma proteobacterium* strain M-4, *Bacillus subtilis* strain Q-1, and *Bacillus* sp. strain E-2 (Sembiring *et al.* 2021). The trial of the application of probiotics through artificial feed found an increase in juvenile sandfish immunity after being challenged with *V. azureus* (Sembiring *et al.* 2020). The dominant probiotics commonly used in aquaculture are the genus *Bacillus* and are mostly exogenous. Then the isolates of these endogenous bacteria must be compared to commercial probiotics whose main composition is *Bacillus* to know their effectiveness as probiotic candidates. Rahman *et al.* (2021) and Cao *et al.* (2022) reported that *Bacillus* strains were highly influential in enhancing growth, immunity, and disease resistance in fish and shrimp. This study aims to determine the endogenous effect isolates of probiotics *G. proteobacterium* strain M-4, *B. subtilis* strain Q-1, *Bacillus* sp. strain E-2 on the growth, digestive enzyme activity, and non-specific immunity of sandfish juveniles compared to commercial probiotics containing *B. subtilis, B. licheniformis*, and *B. pumilus*.

2. Materials and Methods

2.1. Probiotic and Feed Preparation

The combination of endogenous probiotics used was: *G. proteobacterium* strain M-4, *B. subtilis* strain Q-1, and *Bacillus* sp. strain E-2 isolated from the intestines of wild sandfish (Sembiring *et al.* 2021). Each isolate was cultured separately in marine broth media (Himedia-M385) for 48 H and given aeration to stimulate the growth of these bacteria. Commercial probiotics consist of *B. subtilis, B. licheniformis*, and *B. pumilus* used in this study were widely used for shrimp.

Table 1 presents the feed used in this experiment. All ingredients were well mixed, and added water to obtain a moisture content of about 40-50%. Probiotics add at a density of 1×10^7 CFU g^{-1} diet. After all the feed ingredients were mixed, the dough was extruded through a 3 mm die plate to make pelleted feed. The pellets were then air-dried for 3 hours and stored in the refrigerator. Artificial feed manufacturing periodically to avoid a decrease in feed quality.

The diet without probiotics was used as control (A), meanwhile the two treatments were diet with endogenous probiotic isolates consisting of *G. proteobacterium* strain M-4, *B. subtilis* strain Q-1, *Bacillus* sp. strain E-2 (each concentration of 1×10^7 CFU g-1 diet) (B); and diet with commercial probiotic containing *B. subtilis, B. licheniformis*, and *B. pumilus* (1×10^7 CFU g^{-1} diet) (C).

2.2. Feeding Experiment

Healthy juvenile sandfish was obtained from the hatchery of the Research Center for Fisheries-National Research and Innovation Agency-Gondol-Bali. Three hundred sixty sandfish juveniles (initial weight = of 2.72±0.37 g, mean ± standard deviation) were randomly divided into 12 groups and then distributed into 12 plastic containers (59 × 47 × 37 cm^3) at the density of 30 individual/containers. Containers were equipped with a flow-through seawater system at a flow-rate of 1 L min^{-1}. Juvenile sandfish were acclimated to the rearing conditions for one week before the feeding experiment started.

Table 1. Formulation and proximate composition of experimental diets (% of feed)

Ingredients	Concentration
Fish meal	22.00
Ulva meal	10.00
Soybean meal	3.50
Lap lap algae meal	23.25
Rice flour	23.25
Fish oil	2.40
Vitamin mix1)	1.00
Mineral mix2)	1.00
Filler	9.60
Carboxymethyl cellulose (CMC)	4.00
Total	100.00
Moisture (%)	65.53
Crude protein (% dry matter)	20.47
Lipid (% dry matter)	5.10
Ash (% dry matter)	26.71
Fiber (% dry matter)	1.34

[1)]Vitamin mix: Vit A 5,000 UI/g; Vit D3 1,200 UI/g; Vit E 75 UI/g; Vit K3 23.5 mg/g; Vit B1 15 mg/g; riboflavin 20 mg/g; Vit B6 20 mg/g; Vit B12 0.01 mg/g; pantothenic acid 45.1 mg/g; niacin 100 mg/g; folic acid 7 mg/g; biotin 0.2 mg/g, [2)]Mineral mix (mg/g): $CuSO_4 \cdot 5H_2O$ 20; $FeSO_4 \cdot H_2O$ 50; ZnO 200; $Ca(IO_3)2$ 7.5; MnO_2 50; $CoCO_3 \cdot H_2O$ 0.05; Na_2SeO_3 0.8

Research was completely randomized design with three treatments and each with four replications. During the experiment, the water temperature was maintained at 28-29°C, salinity 32-34 ppt, and pH 8.0-8.3. Low-pressure electrical blowers were installed to provide aeration to each tank to maintain dissolved oxygen levels during the experiment. Sandfish juveniles were fed an experimental diet at 2% of body weight per day (16:00) for four months. Each day, food waste and feces were collected by siphoning.

2.3. Growth Performance and Survival Rate

Growth performance was measured by randomly taking ten sandfish juveniles from each container every 14 days during the experiment to determine weight gain and specific growth rate. At the end of the feeding trial, all sandfish juveniles were counted and weighed individually to determine weight gain, specific growth rate, and survival rate. Growth and survival of juvenile sandfish were calculated as follows: Weight gain (WG) (%) = 100*(W2 – W1) / W1; Specific growth rate (SGR) (% d^{-1}) = 100 (ln W2 - ln W1) / T; Survival rate (%) = 100 × (final number of fish) / (initial number of fish); Where W1 is initial weight; W2 is final weight; T is feeding days; WG is wet weight gain.

2.4. Digestive Enzyme Activities

Digestive enzyme activity was analyzed using three individual intestinal samples from each container. The intestine samples were taken at the beginning, the 1st, 2nd, 3th, and 4th months of the experiment. Analysis of protease activity of the intestine samples of sandfish juveniles using the method of Bergmeyer and Grassi (1983), while amylase activity was analyzed using Worthington (1993) method and lipase activity was analyzed using Borlongan (1990) method.

2.5. Expression Gene Related with Immune Response

At the end of the experiment, a challenge test was performed on sandfish juveniles using *V. azureus*. The challenge test was carried out in a 12 L plastic container using eight juveniles from each container of feeding trial and conducted for 96 hours (4 days). *V. azureus* was injected intraperitoneally at a concentration of 10^6 CFU ml^{-1} with 0.1 ml/individual. Juveniles were fed daily during the challenge test and observed every 24 hours for mortality. At the same time, from each container, coelomate was taken from 3 individuals using a 1 ml syringe which gave an anticoagulant. Immune response parameters observed included total coelomate (TCC), expression of immunity genes including Superoxide Dismutase (SOD), Catalase (CAT), and Lysozyme (LZM) activity. Coelomocytes were counted under a light microscope with 400 magnification and converted to cells ml^{-1}.

2.6. Quantitative Real-Time PCR

Total RNA was extracted from coelomocytes using an RNA extraction solution (Genereach Biotechnology Corp. Taiwan). To determine the RNA concentration using GeneQuant pro (Cambridge). SensiFAST cDNA (Bioline, UK) converts RNA to cDNA with a total RNA of 200 ng in each reaction.

Expression patterns of immune-associated genes, including SOD, CAT, and LZM, were quantitatively determined (qPCR) on the step-one PCR System (Applied Biosystems, USA). The primary sequences of immune genes are present in Table 2, and 18sr RNA is an internal control gene (Sembiring *et al.* 2020). The total volume of the qPCR reaction was 20 µL using the Eva-Green® qPCR mix Plus kit (Solis Biodyne, Estonia). Calculation of Ct of the PCR cycle threshold (Ct) of the affected gene tested normalized relative to Ct 18sr RNA (internal control) on the same sample.

Table 2. Primers used in the study (Huo *et al.* 2018)

Gene target	Primer sequence (5'–3')
SOD (superoxide dismustase)	TCTGAAGGAGGGCTGTCAGT AACTACGCCTTGGTGGTCAG
CAT (catalase)	GACACATCCGGGCTCACTAT GAGCCTAAGCCTGAATGCAC
LZM (lysozyme)	AGGCTACTGGCAGGATGCTATT GCGTACCGTGCCATATAA
18sr RNA	CGA GTC GTG GGA GAT TTT T CAC TTT GGC TGC TTT GAA C

2.7. Data Analysis

Statistical analysis was conducted using SPSS 20.0 software. Data on weight gain, specific growth rate, survival rate, digestive enzyme activity, and immune parameters were analyzed using a one-way analysis of variance (ANOVA) at a significance level of 0.05. When a significant effect was observed, a pairwise comparison using the Tukey post hoc test was employed to identify significant differences between treatments. The normality test used the Kolmogorov Smirnov One-Sample Non-Parametric Test, while the homogeneity test used the Levene Test.

3. Results

3.1. Effects of Probiotics on Growth Performance and Survival Rate of Sandfish Juveniles

After four months of feeding, the final weight and weight gain of sandfish juveniles fed with diet B showed a significant higher (P<0.05) compared to diets C and A (Table 3 and Figure 1). The specific growth rate (SGR) in diet A was lower (P<0.05) compared to diet B. But survival rate was not significantly different (P>0.05) among treatments and ranged from 82.08% to 85.42% (Table 3).

3.2. Effects of Probiotics on Digestive Enzyme Activity of Sandfish Juveniles

Sandfish juveniles fed diet B had significantly higher protease activity than those in diets C and A (P<0.05; Table 4). Lipase and amylase activities were higher with diet B compared to sandfish with diet A (P<0.05) but not significantly different from that in feed C (P>0.05).

3.3. Effects of Probiotics on Mortality Rate and Immune Response Based on *Vibrio* Challenge Test

Administration of probiotics in feed enhanced disease resistance of sandfish juveniles against *V. azureus* infection (Figure 2). The cumulative mortality rate at treatment B was 18.75%, significantly lower than treatment C (31.25%) and treatment A (37.50%).

Table 3. The influence of feeding containing probiotics and without probiotics on the growth performance and survival rate of sandfish (*H. scabra*) juveniles during the four months experimental periods

Items	Treatment		
	A	B	C
Final weight (g)	8.33±1.53^{a}	11.16±0.17^{b}	8.75±1.28^{a}
Specific growth rate (% day^{-1})	0.92±0.16^{a}	1.17±0.01^{b}	0.97±0.13^{ab}
Weight gain (%)	206.07±56.19^{a}	310.11±6.36^{b}	221.69±46.94^{a}
Survival rate (%)	82.08±1.60^{a}	85.42±2.85^{a}	83.75±3.44^{a}

Means in the same row with different superscript letters are significantly different at Tukey post hoc test, (P<0.05)

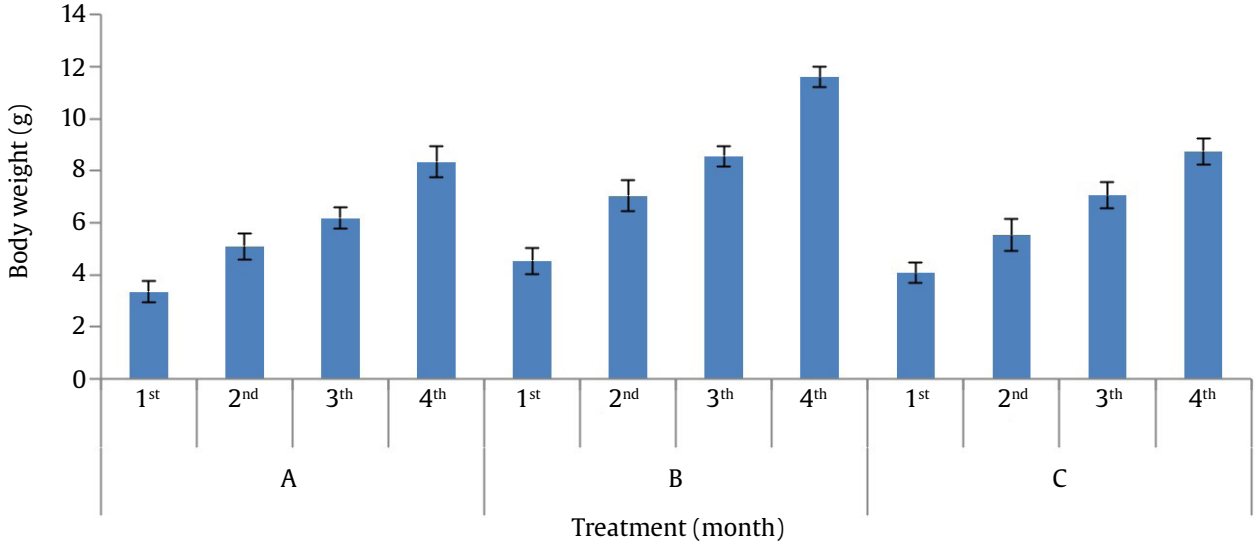

Figure 1. The growth pattern of sandfish (*H. scabra*) juveniles fed diet containing probiotics and without probiotics for four months

Table 4. Influence of feeding containing probiotics and without probiotics on the activities of intestinal digestive enzymes of sandfish (*H. scabra*) juveniles

Treatment	A	B	C
A	2.59±0.03[a]	2.73±0.03[a]	0.81±0.02[a]
B	3.88±0.05[b]	3.45±0.05[b]	1.21±0.02[b]
C	2.69±0.02[a]	3.12±0.03[ab]	0.98±0.03[ab]

Means in the same column with different superscript letters are significantly different at Tukey post hoc test, (P<0.05)

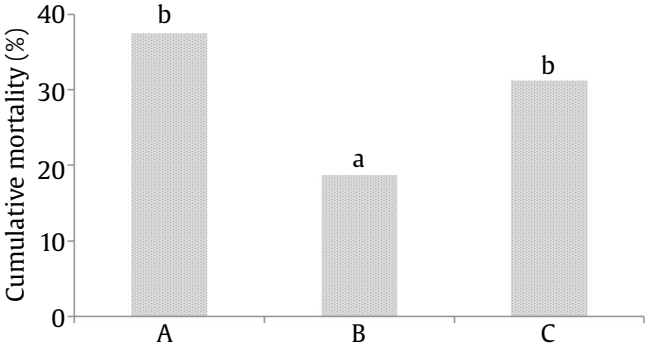

Figure 2. Cumulative mortality of sandfish (*H. scabra*) juveniles after being challenged with *V. azureus*

Juvenile sandfish in treatment B have the highest TCC value. TCC values of sandfish juveniles fed a probiotic-supplemented diet (treatments B and C) significant increase (P<0.05 compared to the control after the challenge test with *V. azureus* (Table 5).

SOD gene expression in juvenile coelomocytes at 24 hours of observation was not significantly different among treatments A, B, and C (P>0.05). While at 48, 72, and 96 hours of observation, SOD gene expression in treatment B was substantially different from treatment C and A (P<0.05) (Figure 3D). CAT and LZM gene expression in treatment B, starting from observation 24 H, showed an increased value and significantly differed from the other two treatments (P<0.05). Meanwhile, at 72 H, there was no significant difference (P>0.05) (Figure 3E and F).

4. Discussion

Significantly better growth performance of sandfish juveniles at treatment B appeared by the second week of the experiment compared to treatment A (without probiotics) in the 2 months (Figure 1). According to Li *et al.* (2015); Kwoji *et al.* (2021), and Puvanasundram *et al.* (2021), applying a combination of different probiotics resulted in an increased survival rate, better growth, inhibited pathogens and showed a synergistic effect compared to the administration of a single probiotic in fish.

The abilities of marine species to absorb and utilize nutrients for growth depend on the activities of digestive

Table 5. TCC of sandfish (*H. scabra*) juveniles challenged with *V. azureus* at the end of the experiment

TCC ($\times 10^4$ ml^{-1})	Treatment		
	A	B	C
0 H	84.00±0.35[a]	135.67±0.27[0]	103.00±0.33[a]
24 H	107.00±0.30[a]	141.33± 0.61[b]	108.33±0.53[a]
48 H	128.00±0.52[a]	182.67± 0.45[b]	167.00±0.50[b]
72 H	105.33±0.68[a]	204.00± 0.36[c]	137.33±0.28[b]
96 H	115.00±0.30[a]	281.67± 0.29[c]	189.33±0.70b

Means in the same row with different superscript letters are significantly different at Tukey post hoc test, (P<0.05)

enzymes, and the enzyme activity is also proven to be closely related to feeding levels (Sun *et al.* 2015). The juvenile sandfish fed a diet containing endogenous isolates probiotics (diet B) showed significantly increased their protease, lipase, and amylase activity. Higher protease and amylase activity can promote the breakdown of proteins and carbohydrates, thereby increasing their digestion and absorption. The high activity of this enzyme explains why the growth of juvenile sandfish increased significantly in treatments B and C compared to treatment A as a control. The results of the present study agree with studies reported by Yang *et al.* (2015) and Zhao *et al.* (2016). They found that applying probiotics in the feed for sea cucumbers showed significantly increased activity of intestinal digestive enzymes, including amylase and trypsin.

Significant high activity of the protease enzyme of juvenile sandfish in treatment B compared to the juvenile sandfish in treatments C and A might be due to the presence of *G. proteobacterium*. (Gao *et al.* 2014; Wang *et al.* 2017), reported that Proteobacteria are bacteria that have an essential role in the intestines of sandfish as protease-producing bacteria.

Coelomocyte is a crucial component of the non-specific immune system and is an essential defense against phagocytosis in sea cucumbers (Chiaramonte and Russo 2015; Rathinam *et al.* 2020). The low resistance of juvenile sandfish after being challenged with *V. azureus* in treatment A-without probiotic and C-with single species caused a decrease in immune response parameters such as TCC, SOD, CAT, and LZM. The survival ability depends on the immunity readiness of the juveniles against infection. These results are in line with Li *et al.* (2015), who reported that using mono-species and multi-species probiotics could increase the immune response of sea cucumber juveniles, as shown by the increase in SOD, CAT, and LZM activity in coelomate.

Feed supplemented with probiotics significantly affected the relative expression of SOD and CAT in the coelomic fluid of juvenile sandfish compared to feed without probiotics (P<0.05). Probiotics enter the gastrointestinal of aquatic organisms in the same way

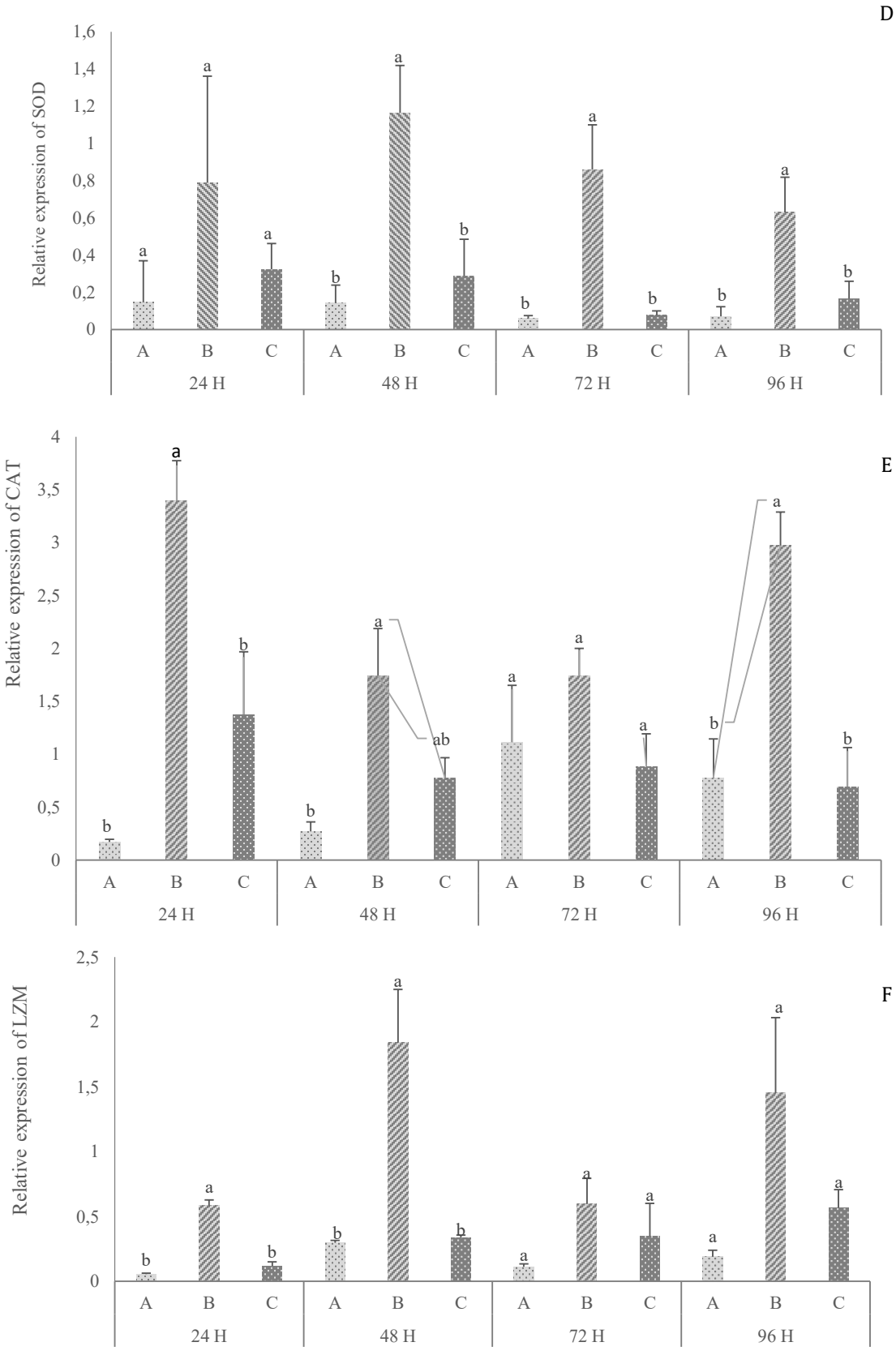

Figure 3. Influence of feeding containing probiotics and without probiotics on the relative mRNA expression of the immune response. (D) SOD, (E) CAT, (F) LZM of sandfish (*H. scabra*) juveniles on the 24H, 48H, 72H, and 96H after challenge test with *V. azureus* strain 4C-1. Treatments with different letters are significantly other (P<0.05)

as they do in terrestrial animals, interfere the pathogen function through the production of antagonistic compounds, competing for substrates and resources such as nutrients, physical space, and even oxygen (Fuller 1989). The increase of coelomocyte capacity by probiotic induction, the ability to phagocyte, entrap and encapsulate invading microorganisms is a valid immune cell mediated mechanism of echinoderms (Chia and Xing 1996).

In Echinodermata, stimulation of phagocytic coelomocytes results in increased uptake of oxygen and generate superoxide anions (O_2), which in turn leads to the production of other highly reactive oxidants that play the roles as microbiocidal and cytotoxic agents (Coteur *et al.* 2002). SOD and CAT are antioxidant enzymes and play an essential role as active oxygen scavengers in eliminating excessive free radicals in the body (Ighodaro and Akinloye 2018). Initially, SOD converts the superoxide radicals to O_2 and H_2O_2 and CAT in the next step transforms the H_2O_2 into O_2 and H_2O (Howcroft *et al.* 2009). Thus, antioxidants play a crucial role in the maintenance of cell integrity, homeostasis, and in prevention of oxidative damage (Dix and Aikens 1993; Vigo-Pelfrey 1990). LZM activity in juvenile sandfish coelomate was also higher in feed B compared to feed C and A. This data proves that combining probiotics can stimulate the sandfish's immune response. LZM as a non-specific immune mediator, has an antibacterial activity by attacking peptidoglycans of bacterial cell walls (Melillo *et al.* 2018; Xue *et al.* 2015). Additionally, as an important hydrolytic enzyme. LZM can disrupt β-(1,4) glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan (Callewaert and Michiels2010; Van-Herreweghe and Michiels 2012).

A diet combined with probiotics *G. proteobacterium* strain M-4, B. subtilis strain Q-1, and *Bacillus* sp. strain E-2 is very beneficial for sandfish juveniles in terms of increasing growth rate and digestive enzyme activity, improving health and disease resistance. The process is by modulating intestinal micro-flora and stimulating the innate immune system. Applying probiotics in the diet can be a new strategy to improve growth and health management in cultivating sandfish.

Conflict of Interest

The authors declare no conflicts of interest in this research.

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