Isolating and characterizing bacteria in the intestine of wild sandfish, Holothuria scabra as probiotics candidate

S B M Sembiring¹, J H Hutapea¹, I N A Giri¹, S Hadisusanto², R Pratiwi² and Haryanti¹

¹ Institute for Mariculture Research and Fisheries Extension, Gondol, Singaraja 81155, Indonesia
² Gadjah Mada University, Indonesia

Email correspondent: moriasembiring@gmail.com

Abstract. The research aims to isolate bacteria as potential probiotics for rearing of sandfish, H. scabra. The procedures were isolating bacteria from nature sandfish’s intestines, characterizing, identifying, enzymatic hydrolysis activity and pathogenic testing, and in vivo testing of candidate probiotics. Identification of probiotic bacteria was based on 16S rRNA encoding gene sequence. Similarity identification was conducted by using BLAST on NCBI. The enzymatic activity test was carried out through Extra Cellular Product (ECP) of isolated bacteria. The in vivo test was done in twelve 1.2 m³ tanks. Initial mean body weight of juvenile was 6.0 ± 4.3 g and total length 4.3 ± 0.6 cm. The results, there were three isolated bacteria as candidates of probiotics with code M-4, Q-1, and E-2 which had high ability in hydrolyzing gelatin, casein, amylase, lecinthin, and lipase enzymes. The M-4 was identified as Gamma proteobacterium with a 99% similarity, Q-1 and E-2 were identified as Bacillus subtilis and Bacillus sp with 98% and 97% similarity. The in vivo trial of probiotic candidate in feed for juvenile gave higher survival rate 95% compare to control 91%. The growth performance was (7.3 ± 2.1 g; 4.5 ± 0.7 cm) in probiotic which higher compare to control (6.0 ± 1.8g; 4.3 ± 0.8 cm).

1. Introduction
The control of bacterial diseases, which was generally conducted by administering antibiotics or chemicals, is not allowed any longer in the aquaculture system. This is because antibiotics can make the pathogenic bacteria become resistant, so that it will have a negative effect on the health of the consumers because antibiotic residue may contaminate water environment. Hence, there is a need to study on alternative environmental-friendly disease control system. The use of probiotics is one of the alternatives that plays a role as a biological control used as a natural enemy of the pathogenic bacteria [1]

Probiotics is living microbe that can be added to the feed and gives a beneficial effect on the host by correcting the balance of the intestine microbe [2]. However, the role of probiotics is not only to improve the host response to the disease but also to improve the quality of the environment [3]. Probiotics often does not do its role directly, but through enzymatic excretion to improve feed nutrition, hinder pathogen, increase the growth and immunity of the body [4].

The use of some bacteria, found in the culture media and in the body of organisms, such as shrimps
and fish as probiotics, has shown a positive effect on the growth and health of the fish through the improvement of the body microbial balance [5]. The effectiveness of probiotic bacteria for controlling pathogenic microorganism is greatly affected by the type of the bacteria used [6]. This is because the bacteria life is strongly influenced by the environment. Another criterion to meet to make certain microorganism probiotics is the fact that the microorganism is not pathogenic and does not produce a compound that is toxic to the cultivated animal [7]. Thus, [8] state that a study on probiotics and sieving method used to get probiotic strain is one of an integrated strategy in controlling diseases and protecting the environment in an aquatic cultivation.

By the increasingly high demand for the sea cucumber commodity and its derivative products (especially food and medical products), then an intensive sea cucumber cultivation is the way to meet the demand. The problems occurring during juvenile rearing process are death because of diseases and the relatively slow growth. Thus, the study about the potential probiotics to increase their survival and growth rate is still required. Some results of the studies using of probiotic in rearing juvenile, A. japonicus to increase its growth, immunity to diseases and to reduce the growth of pathogen have been conducted [9], [10], [1]. Then, [11] have also isolated bacteria from A. japonicus, and based on the enzymatic activity, 3 types of probiotics were obtained, i.e., Bacillus sp. BC26, Pseudoalteromonas sp. BC228 and Vibrio tasmaniensis BC232 that can be used as probiotics. However, those three types of probiotics have not been tested in vivo, in terms of growth and immunity. Study on H. scabra in rearing juveniles using probiotics has never been performed, thus the present study was conducted with the aim of getting probiotics-candidate bacteria through isolating, characterizing, and identifying microorganisms found in sandfish intestine.

2. Materials and methods
This study was conducted in some stages, namely:

2.1. Collecting the sample of sandfish
The sample of sandfish was obtained from a fisherman’s catch around Pajarakan coastal waters, Gerokgak, Buleleng regency, Bali. The sandfish collected were one big size with weight and length of 138.7 g and 12.0 cm, and one small size with 86.3 g and 11.0 cm, consecutively. Then, each of the sandfish was cut and removed its intestine. The intestine sample was cut along, those with sand and the ones without any sand. Each sample was put into a micro tube and was added with one mL sterile seawater, homogenized and filtered (3µm) and then used in the bacteria-isolating phase.

2.2. Isolating sandfish intestine bacteria
The sample initially cut into smallest parts was diluted into $10^2$ and $10^4$ concentration subsequently. 100 mL of the result of the dilution was then inoculated into agar marine medium (MA) and thiosulfate citrate bile salt sucrose (TCBS) agar, afterward, it was incubated at 25°C for 1-2 x 24 hours. After being incubated, the colony with different morphological appearance in color, form, and size was purified 3-4 times in a new MA medium using the method of scratched cup so that single bacteria colony as a pure isolate was obtained. The pure isolate was stored in the MA medium at 25°C to be tested further and grown in Marine Medium Broth (MB).

2.3. Characterizing and identifying bacteria candidate probiotic
The pure isolate obtained was grown in Marine Medium Broth liquid media, centrifuged (12,000 rpm for 5 minutes) and the bacteria pellets formed were characterized at the DNA level. The characterization and identification of the pure isolates were performed with the RAPD (Random Amplified Polymorphism DNA) method. The stages of analysis consisted of DNA extraction of bacteria pure isolates using Presto™ Mini gDNA Bacteria kit (Geneaid) with the standard protocol from the kit. PCR amplification used universal primer 2AAM2 (5’-CTG CGA CCC AGA GCG G-3’) at 45°C in 40 cycles. The electrophoresis of the PCR result was done by using the 2% agarose in 0.5 x SB Buffer. The bacteria isolate and only one isolate to be identified would be taken when there was the
same DNA fragment variation.

The identification of isolated probiotics candidate bacteria was performed by amplifying DNA genome of each isolated bacteria selected based on 16S-rNA gene sequence by sequencing the nucleotide order resulted from the PCR amplification. The PCR used Kit Qiagen (Taq DNA Polymerase kit) in 35 cycles, while the primary pair used was 27 F (5’- AGA GTT TGA TCM TGGG CTC AGP-3’) and 1492 R (5’-ACG GYT ACC TTG TTA CGA CTT-3’). Afterward, the result of the PCR amplification from DNA template of the isolated bacteria was sequenced and compared with the nucleotide bacteria sequence deposited in NCBI (Basic Local Alignment Search Tool) to find out the similarities of the isolated bacteria candidate probiotic.

2.4. Enzymatic hydrolysis activity testing of bacteria candidate probiotic

The result of isolated probiotics candidate bacteria using the pattern of a different DNA fragmentation was undergoing enzymatic activity test through hydrolysis. The hydrolysis enzymatic activity test of the isolated bacteria consisted of: gelatin, casein, amylase, lecithin and lipase enzyme. This enzymatic activity testing began with getting Extra Cellular Product (ECP) of the isolated bacteria that has been cultured using cellophane plat method. The 2% agar media that respectively contain 0.4% gelatin, 0.4% casein, 0.2% starch, 2.5% egg yolk, 1% Tween 80 is an isolated probiotic candidate growing media. Sterile cellophane was put above the surface of the agar media in petri dish and inoculated with bacteria spreading, then it was inoculated for 24 hours at 25°C. The ECP on the surface of the cellophane was washed with buffer PBS liquid, pH 7.0. Then, the obtained suspension was centrifuged for 10 minutes at 13,000 rpm at 4°C. The obtained supernatant was filtered steriley with a 0.22 µm membrane filter.

The enzymatic hydrolysis activity testing method was applied by putting polyethylene cylinder plat above each of the agar media, filled with the 200 µl of ECP liquid and incubated at 37°C for 96 hours. The observation was made at a 24-hour intervals, a clear area around the cylinder plat was the indicator of the enzymatic hydrolysis activity of each isolated bacteria candidate probiotic; the larger the clear area, the more active the enzyme hydrolysis force on the agar substrate of each enzyme source content.

2.5. Pathogenic test of probiotics candidate bacteria

The pathogenetic testing was conducted on the juvenile sandfish by using isolated bacteria with a high activity to hydrolyze the enzyme. The average weight and length of the juvenile were 15.37 ± 3.06 g; 5.58 ± 0.66 cm (Table 1). Each of probiotics candidate bacteria was cultured in Marine Broth media, then incubated at 20°C by using a shaking incubator for 24 hours. The pathogenicity testing was done through a 100 µL intra-muscular injection of the sandfish. Each isolated bacteria was tested twice with the average of 10^7 CFU/mL concentration (Table 1).

| No | Average Total Length (cm) (n= 5 ind.) | Average Body Weight (g) | Isolated Bacteria | Bacteria Concentration (CFU/mL) |
|----|-------------------------------------|-------------------------|------------------|--------------------------------|
| 1  | 5.68 ± 0.82                        | 14.93 ± 3.14            | E-2WS            | >10^7                           |
| 2  | 5.61 ± 0.61                        | 15.77 ± 2.32            | I-4CS            | 4.3 x 10^9                      |
| 3  | 5.32 ± 0.52                        | 15.51 ± 4.27            | M-4CS            | >10^8                           |
| 4  | 5.46 ± 0.72                        | 14.90 ± 3.07            | Q-1WS            | 1.02 x 10^9                     |
| 5  | 5.60 ± 0.66                        | 14.95 ± 2.76            | C-3CS            | 1.18 x 10^9                     |
| 6  | 5.78 ± 0.69                        | 16.15 ± 3.06            | N-2WS            | 1.02 x 10^9                     |

The juvenile sandfish was reared in twelve 25 L plastic containers, 5 individual per container and two repetitions for each isolate. The pathogenicity of the isolated probiotics candidate bacteria was observed through the mortality of juvenile every 24 hours and was monitored up to 96 hours (4 days)
of rearing. At the end of the rearing, the juvenile survival rate was counted. The survival rate was calculated using Effendie formula (1979).

\[
SR = \frac{N_t \times 100 \%}{No}
\]

Where: \(SR = \) Survival (%), \(No = \) the number of sea cucumbers at the beginning of the experiment, \(N_t = \) the number of sea cucumbers at the end of the experiment

2.6. The in vivo Testing of Bacteria Candidate Probiotic for Rearing the Juvenile of Sandfish through Feed

The cultured bacteria with a probiotic capacity was then mixed with pellet feed at \(10^9\) CFU/mL concentration. The ingredients for the pellet feed are \(Sargassum\) sp meal (30%), \(Ulva\) sp meal (35%), rice flour (18%), soybean meal (4%), fish meal (3%), Lap lap meal (6%), fish oil (1%), vitamin mix (1%), mineral mix (1%) and binder (CMC) (1%).

In the in vivo testing, the juvenile sandfish was provided with pellet feed initially mixed with isolated probiotics candidate bacteria (A) and (B) pellet feed without probiotic (control). Each treatment was repeated 6 times. The rearing containers used were twelve 1.2 m\(^3\) tank. The average weight and length of the juvenile were 6.0 ± 1.4 g and 4.3 ± 0.6 cm, with a 100 juveniles per tank. The testing was done for 40 days and the dosage of feeding was 3% of the total body weight given once in a day (every evening). Every 2 weeks, observation of the growth was made and at the end of the in vivo testing the total number of juvenile was counted to calculate the survival rate.

3. Results and Discussion

3.1. Isolate bacteria candidate probiotic

Probiotic bacteria are bacteria with a favorable role in the intestine and produce harmless substances for the host, but reduce the population of pathogenic bacteria present in the digestive system [12]. Dominant bacteria in the intestine can influence the growth and development of sandfish. The bacteria present in the sandfish intestine produce enzymes helping the digestion process so that the nutrition of feed can be used more efficiently. Furthermore, [13] stated that bacteria play an important role on the organism's health through various ways, including the effect on the morphology and digestive system, feed nutrition efficiency, prevention of pathogen and increase in immunity. There are some conditions to meet by bacteria to be a probiotics candidate, including the fact that the bacteria have anti-microbrial and anti-carcinogenic activities, capable of making a colony in the intestine, and the capacity to increase intestine intake.

In order to get probiotics, isolation of bacteria such as total bacteria and Vibrio obtained from sandfish intestine in the MA, TCBS medium using the spread plate method was done, and the results were shown in Table 2.

Among the total 837 bacteria colonies obtained, there were 124 colonies of pure isolates as probiotics candidate bacteria. The appearances of the isolate colonies varied in MA medium. The colors were cream, white, milky white, and transparent white and the colony forms were oval, small rounded, large rounded, and some were swarming.

| Sandfish sample | Body weight (g) | Total length (cm) | Intestine condition | Total Bacteria CFU/mL | Total Vibrio CFU/mL |
|-----------------|-----------------|-------------------|---------------------|-----------------------|--------------------|
| 1               | 86.3            | 11.0              | With sand           | \(32 \times 10^4\)     | \(322 \times 10^2\) (Y) |
|                 |                 |                   |                     | \(45 \times 10^4\)     | \(198 \times 10^2\) (Y) |
|                 |                 |                   | Without any sand    | \(168 \times 10^3\)    | \(13 \times 10^2\) (Y)   |
|                 |                 |                   |                     | \(284 \times 10^3\)    | \(2 \times 10^2\) (Y)    |

Table 2. Total bacteria and Vibrio from sandfish, \(H. scabra\) intestine.
2nd International Conference on Fisheries and Marine IOP Publishing
IOP Conf. Series: Earth and Environmental Science 890 (2021) 012023 doi:10.1088/1755-1315/890/1/012023

|   |   |   |
|---|---|---|
| 2 | 138.7 | 12.0 |
|   | With sand |   |
|   | 12 x 10^3 | 172 x 10^2 (Y) |
|   | 172 x 10^3 | 209 x 10^2 (Y) |
|   | 115 x 10^2 | 13 x 10^2 (Y) |
|   | 9 x 10^4 | 6 x 10^2 (Y) |
|   |   | 1 x 10^2 (G) |

Remarks:
G: The green vibrio colony
Y: The yellow vibrio colony

3.2. Characterization of probiotics candidate bacteria
Isolated bacteria were purified and then undergone screen test using fingerprint method to get DNA fragmentation patterns of each isolated bacterium. The molecular characterization using the RAPD method with primer 2AAM; of 124 pure isolates of colonies produced only 6 different isolates (Figure 1). Those 6 isolates have different DNA diversity profiles, namely E-2; Q-1; I-4; N-2; M-4 and C-3. Then, those 6 isolates were subjected to hydrolysis enzyme capacity test and pathogenicity test.

![Figure 1. Fragmentation pattern of DNA from isolated bacteria obtained from screening of sandfish, *H. scabra* intestine bacteria (E-2, Q-1 & N-2 were isolated from sandfish intestine without sand; C-3, I-4 & M-4 were isolated from sandfish intestine with sand).](Image)

3.3. Enzymatic hydrolysis activity of probiotics candidate bacteria
Amylum (carbohydrate) and casein (protein) hydrolysis test were performed to find out the capacities of amylase and protease enzyme of the isolated bacteria in analyzing starch and casein. This test was intended to obtain potential bacteria as probiotics candidate for sandfish culture. The enzymatic activity tests were done for gelatin, casein, amylase, lecithin, and lipase. The existence of transparent zone mark around ECP of the tested isolates showed that the bacteria are able to hydrolyze the enzyme tested. The basic principle of probiotic mechanism is the capacity of microorganisms in breaking or hydrolyzes the long chain carbohydrate, protein, and fat in feed given. Such ability is derived from microbes because of the presence of special enzymes to break these bonds.

The diameter of hydrolysis activity was illustrated in Figure 2. Based on measurement of hydrolysis diameter for gelatin, there were three isolates formed 55-70 mm diameter M-4, Q-1 and E-2. Isolate with highest ability to hydrolyze casein was E-2 with diameter of 20 mm, then followed by Q-1 with diameter of 17.5 mm and isolates with highest ability to hydrolyze amylase was E-2 with diameter of 15.5 mm. Then, isolates with highest ability to hydrolyze lecithin were I-4, N-2 with diameter 1.4 – 1.6 mm and for lipase was E-2 with diameter of 5-6 mm. Those isolates bacteria were tested further for the probiotic selection. The ability of isolated bacteria to hydrolyze protease, lipase and amylase means those bacteria are able to utilize gelatin, casein, fat and amylase as energy sources and carbon. All isolates bacteria are able to hydrolyze gelatin and casein, meaning that the isolates have protease enzyme activity or able to
prevent protein to become peptide or amino acid. According to [14], the hydrolysis of protein into single amino acid with the aim of using the amino acid to synthesize new protein and other cellular molecules or to become an energy source.

![Enzymatic hydrolysis test of isolated bacteria as probiotics candidate](image)

Figure 2. Enzymatic hydrolysis test of isolated bacteria as probiotics candidate (a: gelatin; b: casein; c: amylase; d: lecithin; e: lipase)

### 3.4. Pathogenicity testing of bacteria candidate probiotic

Pathogenicity test was performed to find out whether isolated bacteria is pathogenic or not to the juvenile sandfish. Tests were performed for 96 hours on the six isolates in which formed high hydrolysis zone (E-2, Q-1, I-4, N-2, M-4 and C-3) and the results are shown in Table 3. Isolated bacteria did not cause any mortality and the occurrence of pathogenic infection, meaning that the isolates are safe and nontoxic. Furthermore, it could be stated that all probiotics injected intramuscularly were not pathogenic to the sandfish juvenile.

| Isolated Bacteria | Number of sandfish survived until 96 hours after injected with isolated bacteria (individual) | Survival rate (%) |
|-------------------|-----------------------------------------------|-------------------|
|                   | Initial | 24 hours | 48 hours | 72 hours | 96 hours |                   |
| E-2               | 5       | 5        | 5        | 5        | 5        | 100               |
| Q-1               | 5       | 5        | 5        | 5        | 5        | 100               |
| I-4               | 5       | 5        | 5        | 5        | 5        | 100               |
| N-2               | 5       | 5        | 5        | 5        | 5        | 100               |
| M-4               | 5       | 5        | 5        | 5        | 5        | 100               |
| C-3               | 5       | 5        | 5        | 5        | 5        | 100               |

![Table 3](image)
3.5. Identification of bacteria molecularly

Based on nucleotide sequence analysis of the 6 bacteria isolates after being blasted, 4 isolates were *Bacillus* sp. (E-2; Q-1; I-4; C-3), one belonged to *Proteobacterium* (M-4) with a high value of similarity (97-99%) and one isolate (N-2) was *Staphylococcus cohnii* with only 97% similarity with DNA sequence in each accession number (Table 4).

The result of sequencing of the 6 (six) bacteria isolates showed that 80.65% of the isolates belongs to the genus *Bacillus* (Table 4). [15], state that *Bacillus* sp. plays a role as probiotic bacteria capable of breaking feed relatively quick and reduce pathogenic bacteria present in the intestine and the bacterial activity in the intestine will change quickly when microbes enter it through feed and water. Similarly, [16] states the *Bacillus* sp. has the ability to secret protease, lipase and amylase enzymes.

As probiotics, research on *Bacillus* spp. was plenty due to its ability on attach and to produce bacteriosine (peptide anti-microbe) and immunostimulant [17]. This strain was proofed as commercial probiotic product and able to increase shrimp production as well as be used as anti-microbe [18]. *Bacillus* spp. also has another advantage since its spores could be stored [19].

Table 4. Similarity sequence of DNA isolated from isolate bacteria as probiotic candidate for sandfish, *H. scabra* to sequence on GenBank from each accession.

| No | Isolate | Species Name                | Similarity (%) | No.Accession     |
|----|---------|------------------------------|----------------|-----------------|
| 1  | E-2     | *Bacillus subtilis*          | 97             | KP289263.1      |
| 2  | Q-1     | *Bacillus subtilis*          | 98             | MH114036.1      |
| 3  | I-4     | *Bacillus subtilis*          | 97             | KF830992.1      |
| 4  | N-2     | *Staphylococcus cohnii*      | 97             | KX631432.1      |
| 5  | M-4     | *Gamma Proteobacterium*      | 99             | HE576774.1      |
| 6  | C-3     | *Bacillus subtilis*          | 98             | KY623224.1      |

The results of studies related to probiotic characters in sandfish showed that the micro biota characterization in the intestine of *Holothuria glabelrrima* done by [20] other than the class *Gamma proteobacteria, Bacillus* sp. was also found. The same result was also found from the study conducted by [21] in the sandfish *A. japonicus*. Then, to increase the immunity of juvenile sandfish, some studies have been done by adding probiotics to the feed [22], [23], [24] also state that *Bacillus* is a bacterium found mostly in animal’s intestine and some genus function as probiotics for prevention against pathogenic attack and increasing the immunity in the cultivated organisms.

The result of isolating the intestine sample with sand showed that there were bacteria from the phylum Proteobacteria (class of Gammaproteobacteria). Some researches toward sandfish (*A. japonicus*) showed that Gammaproteobacteria is a dominant class obtained from the result of isolating sandfish intestines from the catch from the sea and cultivation and from the habitat (sediment substrate) [25], [26] also state that Proteobacteria are the bacteria with important roles in the sandfish intestine, since the class Gammaproteobacteria is reported as bacteria producing protease and can be obtained in the habitat of the sea environment as in sediment and sea water [27]. Although Proteobacteria are abundant, in the recent study, the number was relatively low; however, the isolates can be used as probiotics candidates since the result of their enzymatic activity showed that the isolates could hydrolyze all enzymes used.

3.6. In vivo test of probiotic candidate bacteria through feed in rearing sandfish juvenile

Figure 3 below shows the increasing sandfish growth after being fed by probiotics candidate bacteria-added feed compared to control feed.
Figure 3. The pattern of the increase in total length and body weight of sandfish, H. scabra juvenile by feeding with pellet added with probiotics candidate bacteria and without probiotic as control

In vivo test for 40 days of rearing showed that the total length and body weight of the juvenile fed with pellet added with probiotics candidate bacteria better than fed without the addition of bacteria (control). T-test analysis showed significant difference in the total length ($P<0.05$). Survival rate of juvenile at the end of the in vivo test showed a significant difference between juvenile fed with probiotic (95.3%), while fed without probiotic control was only 91.0%.

Probiotics candidate bacteria mixed in artificial feed for rearing the juvenile sandfish showed a positive response to growth and survival rate. This was caused by the fact that probiotic bacteria can produce intestine enzymes that help the sandfish to digest and use nutrition from the feed given [28]. This shows that addition of probiotics candidate bacteria in pellet feed can be absorbed and digested better when compared to the pellet feed without the giving of the probiotic. Hence, based on the positive response to the growth of the juvenile sandfish from the use of the combination of Gammaproteobacterium bacteria (isolate M-4), Bacillus subtilis (isolate Q-1) and Bacillus sp. (isolate E-2) as probiotics candidate, then the three bacteria isolates can be used as probiotics in rearing sandfish juvenile.

4. Conclusions
Gammaproteobacterium (isolate M-4), Bacillus subtilis (isolate Q-1) and Bacillus sp. (isolate E-2) isolated from sandfish intestine is a potential probiotics candidate for rearing sandfish.

Acknowledgements
The Indonesian Government Fund FY2018 at the Institute for Mari culture Research and Fisheries Extension (IMRAFE) funded this research. This research was conducted with supports from the Sandfish Hatchery and Biotechnology Laboratory. We would like to thank to Made Buda, I Nengah Gede Suparta, Ahmad Rifai, Luh Yuliani Dewi, Luh Tati Aryani and I Nengah Suriadnyani for their technical support.

References
[1] Chi C, Liu J Y, Fei S Z, Zhang C, Chang Y Q, Liu X L and Wang G X 2014 Effect of Intestinal Autochthonous Probiotics Isolated from The Gut of Sea Cucumber (Apostichopus japonicus) on Immune Response and Growth of A. japonicus. Fish Shellfish Immunol. 38: 367-373.
[2] Sornplang P and Sudthidol P 2016 Probiotic Isolates from Unconventional Sources. Journal of Animal Science and Technology 58-26. 11 p. DOI 10.1186/s40781-016-0108-2.
[3] Nelson L, Blair B and Murdock C 2010 Molecular Analysis of Gut Microflora in Captive-Raised Sea Urchins (Lytechinus variegatus). J. World Aquacult. Soc. 41: 807–815.
[4] Naya S K 2010 Role of Gastrointestinal Microbiota in Fish. Aquacult Res 41: 1553–1573.
[5] Villamil L, Figueras A, Planas M and Novoa B 2003 Control of Vibrio alginolyticus in Artemia Culture by Treatment with Bacterial Probiotics. Aquacult 219:43-56.
[6] Verschure L, Rombaut G, Sorgeloos P and Verstraete W 2000 Probiotic Bacteria as Biological Control Agents in Aquaculture. Microbiol. Mol. Biol. Rev. 64: 655-671.
[7] Fuller R 1992 Histology and Development of Probiotic. In Fuller (Editor): Probiotic the Scientific Basis. Chapman and Hall, p 1-8.
[8] Kesarodi-Watson A, Kaspar H, Josie Lategan M and Gibson L 2008 Probiotics in Aquaculture: The Need, Principles and Mechanisms of Action and Screening Processes. Aquacult. 274: 1-14.
[9] Zheng F, Liu H, Sun X, Qu L, Dong S and Liu J 2012 Selection, identification and application of antagonistic bacteria associated with skin ulceration and peristome tumescence of cultured sea cucumber Apostichopus japonicus (Selenka). Aquacult. (334-337):24-29.
[10] Zhao Y, Zhang W, Xu W, Mai K, Zhang Y and Liufu, Z 2012 Effects of potential probiotic Bacillus subtilis T13 on growth, immunity and disease resistance against Vibrio splendidus infection in juvenile sea cucumber Apostichopus japonicus, Fish Shellfish Immun. 32 (5): 750–755.
[11] Yang Z P, Sun F X, Liu Z M, Zhang L, Cao W and Ma Y X 2013 Screening and identification of potential enzyme producing probiotics from gut sea cucumber Apostichopus japonicus. J. Dalian Fish Univ. 28: 17-20. (in China, with English abstract).
[12] Flores M L 2011 The use of Probiotic in Aquaculture: an Overview. Int Res J Microbiol 2(12):471-478.
[13] Robertson P A W, O’Dowd C, Burrells C, Williams P and Austin B 2000 Use of Carnobacterium sp. as a probiotic for atlantic salmon (Salmo salar L.) and rainbow trout (Oncorhynchus mykiss, Walbaum). Aquacult. 185: 235-43.
[14] Kaiser C, Merwe R V D, Bekker T F and Labuschange N 2005 In-vitro Inhibition of Mycelial Growth of Several Phytopathogenic Fungi, Including Phytophthora cinnamomi by Soluble Silicon. South African Avocado Growers Association Yearbook 28: 70-74.
[15] Anggriani R, Iskandar and Taofiqurohman A 2012 Effectivity of commercial feed added with probiotic Bacillus sp. isolated from digestive tract of Pangasius on survival and growth of Red tilapia (Oreochromis niloticus). Journal of Marine and Fisheries 3(3):75-83.
[16] Febryana M 2017 Isolation and identification of Bacteria from Common carp (Cyprinus carpio) intestine. Thesis. Waters Sources Management. Agriculture Faculty. North Sumatera University 51 p.
[17] Barbosa T M, Serra C R, La Ragione R M, Woodward M J and Henriques A O 2005 Screening for Bacillus isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 71(2):968-978.
[18] Decamp O and Moriarty D J W 2006 Probiotics as alternative to antimicrobials: limitation and potential. World Aquacult. 37(4): 60-62.
[19] Hong H A, Duc L H and Cutting S M 2005 The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29: 813-835.
[20] Jimenez M P, Ruiz-Calderon J F, Dominguez-Bello M G and Garcia-Arrara J E 2018 Characterization of The Intestinal Microbiota of The Sea Cucumber Holothuria glaberrima. PLOS ONE available from: https://doi.org/ 10.1371/journal.pone.0208011. 16 pp.
[21] Enomoto M, Nakagawa S and Sawabe T 2012 Microbial communities associated with holothurians: presence of unique bacteria in the coelomic fluid. Microbiol Environ 27: 300-305.
[22] Zhao Y, Mai K, Xu W, Zhang W, Ai Q, Zhang Y, Wang X and Liufu Z 2011 Influence of dietary Probiotic Bacillus TC22 and Prebiotic fructooligosaccharide on Growth, Immune Responses and Disease Resistance Against Vibrio splendidus Infection in Sea Cucumber Apostichopus japonicus. J. Ocean Univ. China 10(3): 293-300.
[23] Yan F, Tian X and Dong S 2014 Effect of Bacillus baekryungensis YD13 Supplemented in Diets on Growth Performance and Immune Response of Sea Cucumber (Apostichopus japonicus). J. Ocean Univ. China 13(5): 805-810.
[24] Gong K, Wang B J, Liu M, Jiang K Y, Qiu C W, Luo Z Y, Fan R Y and Wang L 2012 The Influence of Lactic Acid Bacteria and Metabolites on Intestinal Microflora and Nonspecific Immunity of Juvenile Sea Cucumber (Apostichopus japonicus). Marine Sci. 32(7):7–12. (in Chinese with English abstract).

[25] Gao F, Li F H, Tan J, Yan J P and Sun H L 2014 Bacterial Community Composition in the Gut Content and Ambient Sediment of Sea Cucumber Apostichopus japonicus Revealed by 16S rRNA Gene Pyrosequencing. PLoS One, 9(6): e100092. https://doi.org/10.1371/journal.pone.0100092 PMID: 24967593.

[26] Li X M, Zhu Y J, Yan Q Y, Ringo E and Yang D G 2014 Do the Intestinal Microbiotas Differ Between Paddlefish (Polyodon spathala) and Bighead Carp (Aristichthys nobilis) Reared in The Same Pond. J. Appl. Microbiol. 117: 1245-1252.

[27] Wang Q Z, Cui Y, Sen B, Ma W M, Zheng R L, Liu X H and Wang G Y 2017 Characterization and Robust Nature of Newly Isolated Oleaginous Marine Yeast Rhodosporidium spp. from Coastal Water of Northern China. AMB. Express 7: 30. available from: https://doi.org/10.1186/s13568-017-0329-x

[28] Widanarni, Nopitawati T and Jusadi D 2015 Screening of Probiotic Bacteria Candidates from Gastrointestinal Tract of Pacific White Shrimp, Litopenaeus vannamei and Their Effect on The Growth Performances. Res. J. Microbiol. 10(4):145-157.