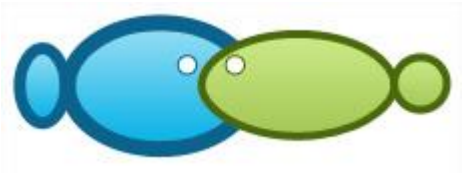

Application of a consortium of bacterial symbionts, contained in the sea cucumbers' stomach, as dietary adjuvant and its potential for marine cultivation

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Abstract. The sea cucumber stomach's bacterial consortium, containing symbiont bacteria, has prospects in the field of aquaculture due to its potential antibacterial properties. This research aimed to determine the proximate content of the dietary mixture of the sea cucumber gut bacteria, the number of bacteria in the fish medium and their effect on the water quality in tilapia cultivation. A bacterial consortium from the sea cucumber stomach's content is processed in the form of bran flour, then mixed with fish feed in different percentage ratios: treatment A, with 75% pellets and 25% bacterial consortium; treatment B, with 50% pellets and 50% bacterial consortium; treatment C, with 25% pellets and 75% bacterial consortium. The results showed that the proximate content of the consortium of bacteria in the stomach content of sea cucumbers was rich in crude fiber (27.62) and carbohydrates, non-nitrogenous organic matter and nitrogen-free extract (37.64). The medium salinity, temperature and pH during the test ranged between 8 and 10 ppt, 23.50 and 25.00°C, and 7 and 7.98, respectively. The results showed that the lower the concentration of the bacterial consortium (25%), the better the fish growth. The consortium of bacteria in the stomach content of sea cucumbers can reduce the number of bacteria (CFU mL^{-1}) in from the fish medium. They contain phthalate compounds. In conclusion, the consortium of sea cucumber gut bacteria has the potential to be antibacterial in tilapia treatment.
Key Words: bacterial consortium, fish, growth, GCMS, proximate, phthalate compounds.

Introduction. Sea cucumbers have become a target for useful bioactive compounds in the world of marine pharmaceuticals (Santosa et al 2020). The presence of bacteria associated with the stomach contents of sea cucumbers has made possible the use of these organisms as the main source of new bacteria and a source of bioactive compounds. Symbiotic bacteria in the stomach contents of sea cucumbers have been found to have antibacterial activity. They include the bacterium *Bacillus toyonensis* strain BCT-7112, *Bacillus aquimaris* strain TF-12, *Bacillus maritimus* strain KS 16-9 and *Virgibacillus chiguensis* strain NTU-101 (Pringgenies et al 2018). The four types of bacteria have synergistic characters (Girsang et al 2020).

B. toyonensis bacteria have been used in animal nutrition in several parts of the world (Okaiyeto et al 2015). *B. aquimaris* bacteria are candidate bacteria as a source of bioactive surfactants, which are substances that have the ability to reduce the surface tension of a medium and reduce the interfacial tension between two phases, with different degrees of priority. Moreover, these bacteria have compounds that encode the enzymatic activity of reducing the molecular weight of polysaccharide compounds, as well as the osmotic stress response. *B. maritimus* bacteria are tolerant to temperature fluctuations in the range of 12 to 42°C (optimal value is 30°C), regarding their growth (Pal et al 2017). Meanwhile, *V. chiguensis* bacteria are Gram-positive and have flagella for their movement and white bacteria.

Feed is one of the important factors that affect the growth and livelihoods of fish that will be cultivated (Eriegha & Ekokotu 2017; Kong et al 2020). Tilapia is consumed by almost all Indonesian people and can be found on traditional markets. The mixture of fish feed with a consortium of bacteria is expected to have the potential to increase fish survival due to their antibacterial properties, which prevent the water contamination and the infections during its growth. The purpose of this study was to determine the proximate content of the dietary mixture of the sea cucumber gut bacteria, the number of bacteria in the fish medium and their effect on the water quality in the tilapia cultivation, as well as determining the bacterial consortium of the cucumber stomach using the chromatography method.

Material and Method

Materials. The test fish used in this study were saline tilapia (*Oreochromis niloticus*) with a size of 4-6 cm with an average weight of 170 g, from a total of 60 fish specimens by container, originating from Siwarak, Semarang, Central Java. The test fish were adapted for 7 days in a container filled with water and were given pellets twice a day with the *at satiation* method (until they were full), which was marked by the behavior of the fish avoiding the feed. The culture medium was aerated during the observation period (42 days).

Research design. The research design used was a completely randomized design (CRD) with 3 treatments and 3 repetitions carried out for 42 days. The experiment consisted of: treatment A, with 75% pellets with and 25% bacterial consortium pellets; treatment B, with 50% pellets with and 50% bacterial consortium, and treatment C, with 25% pellets with and 75% bacterial consortium.

The test feed included the mixture of commercial pellets added with the bacterial consortium mixture. The feed used in this research was commercial fish feed in the form of pellets with a diameter of 0.5-0.7 mm. To mix the pellets and the consortium, the bacterial isolates were purified, using the streak method until pure cultures were obtained. The bacterial consortium isolates were collected from the laboratory of the Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang. The consortium of sea cucumber gut bacteria as a mixture of fish feed was made from bran, using the modified method of Pringgenies et al (2016). First, the four bacterial isolates, namely *B. toyonensis* strain BCT-7112, *B. aquimaris* strain TF-12, *B. maritimus* strain KS 16-9 and *V. chiguensis* strain NTU-101 were mass cultured with liquid zobell media for 1x24 h. Then the results of the bacterial mass culture were added in a solution of Peton, beef extract, aquades. Peton, beef extract, aquades were mixed first until homogeneous and then placed into a 300 mL Erlenmeyer, sterilized in an autoclave for 15 minutes. Then the mixture was cooled and then incubated while being shaken with a rotation of 150 rpm for two days to be mixed with the bran. Then, 1 kg of bran was weighed, then the bacteria (*B. toyonensis, B. aquimaris, B. maritimeus* and *V. chiguensis*) was measured. The ratio of bacteria and bran was 1 kg of bran for 1.5 L of bacteria ($105x10^6$ CFU mL^{-1}). The two ingredients were mixed until they were homogeneous to form a paste. After the dough is even and homogeneous, it was stored in a tray and flattened thinly to dry quickly. Then the samples were dried in the sun. The dry sample was crushed and stored as test feed. Then, the commercial pellet feed was mashed and mixed with the bacterial consortium according to the ratios tested in the study, then the resulting pellets were dried in an oven at 35°C.

Implementation. The culture medium was water with a salinity of up to 10 ppt, continuously aerated. The medium was filtered. The culture container used was a 20 L plastic bucket filled with 12 L of culture medium. Before the treatment, tilapia was first acclimatized in the container for 7 days and they were given pellets twice a day at satiation. After that, the fish were weighed to determine the initial stocking weight, and then stocking was carried out in each container with a stocking density of 5 individuals per container. Tilapia treatments were carried out for 42 days. Feeding was given 2 times

a day at 09.00 and 16.00 WIB at a satiety level (when fish are full, which is indicated by keeping the fish away from the feed given).

Growth sampling was carried out every 7 days. The weighing was performed using an electric scale. Fish length measurements were carried out using millimetre blocks and a ruler, from the beginning until the end of rearing. The water quality management was carried out by measuring water quality parameters such as degree of acidity (pH), salinity and temperature. The water temperature measurement was carried out every day, while the pH, salinity and dissolved oxygen measurements were carried out every 7 days.

Data collections. Data variables observed in this research consisted of Relative Growth Rate (RGR), Feed Consumption Rate (FCR), Protein Efficiency Ratio (PER), Feed Utilization Efficiency (FUE) (Pereira et al 2007; Bhilave et al 2012).

Relative Growth Rate (RGR):

$$RGR = [(Wt - Wo)/ Wo \times t) \times 100]$$

Where:
Wt - biomass at the end of the study (g);
Wo - biomass at the beginning of the study (g);
t - Length of study (days).

Feed Consumption Rate (FCR):

$$FCR = [F / (Wt + d) – Wo]$$

Where:
F - the amount of feed consumed;
Wt - fish biomass at the end of the study (g);
Wo - fish biomass at the beginning of the study (g);
D - fish biomass that died during the study (g).

Protein Efficiency Ratio (PER):

$$PER = [(Wt - Wo)/ Pi) \times 100]$$

Where:
Wt - fish biomass at the end of the study (g);
Wo - fish biomass at the beginning of the study (g);
P - the protein weight of the feed consumed (g).

Feed Utilization Efficiency (FUE):

$$FUE = [(Wt–Wo)/ F) \times 100]$$

Where:
Wt - weight at the end of the study (g);
Wo - weight at the beginning of the study (g);
F - the amount of feed consumed during the study (g).

Data analysis. The proximate analysis included moisture, ash, fiber, protein and fat content in the fish feed that has been added with a bacterial consortium using the Kjedahl A0AC 2001.11 method. Physical and chemical water quality parameters observed in this study were: temperature, pH, salinity and the number of bacteria. Measurement of water temperature was carried out every afternoon before feeding, while pH and salinity measurements were carried out every 7 days.

Water samples of 1 mL were taken from the fish medium and put into a test tube containing 9 mL of sterile seawater for gradual dilution. About 1 mL of bacterial suspension was taken from the first test tube, then it was inserted into the second test tube containing 9 mL of sterile seawater, so that a 10^{-2} level dilution was obtained. Dilution was done up to 10^{-5}. The diluted bacterial suspension was planted on Zobell 2216 E medium with the spread plate method, by taking 100 µL from the surface of each medium, at dilutions of 10^{-3}, 10^{-4}, and 10^{-5}. The bacterial suspension was leveled using a spreader so that the bacteria grew evenly on the surface of the medium, then it was put

in the incubator. Incubation was carried out at 37°C for 2x24 h. Bacterial colonies growing on the medium were counted using the Total Plate Count technique. Bacterial colonies on Petri dishes were counted using a counter. The calculation of the number of bacteria in each Petri dish is expressed in CFU (Colony Forming Unit), as in Sieuwerts et al (2008) and Khan et al (2018).

Gas Chromatography-Mass Spectrometer (GC-MS) analysis. GC-MS analysis was done using the GCMS-QP2010S Shimadzu with the following parameters: a 30 m Rtx-5MS column, a 0.25 mm diameter, a programmed temperature from 80 to 300°C with an increment of 10°C min^{-1} and a pressure of 22 kPa in the Helium carrying gas. The number of compounds contained in the extract was indicated by the number of peaks on the chromatogram, while the names/types of compounds were interpreted based on the spectral data of each peak, using the library approach method from the GC- MS database (Johnsen et al 2017).

Results and Discussion. The RGR rates for each treatment are shown in Figure 1.

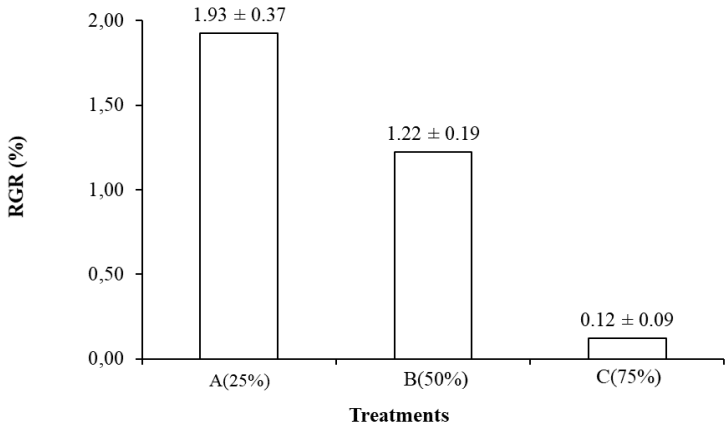

Figure 1. RGR rates of *Oreochromis niloticus* seeds given artificial feed with different amounts of feed contents for 35 days of treatment, for each treatment (A, B, C).

Feed Consumption Rate (FCR). The FCR results of *O. niloticus* seeds given artificial feed with different amounts of bacterial content can be seen in Figure 2.

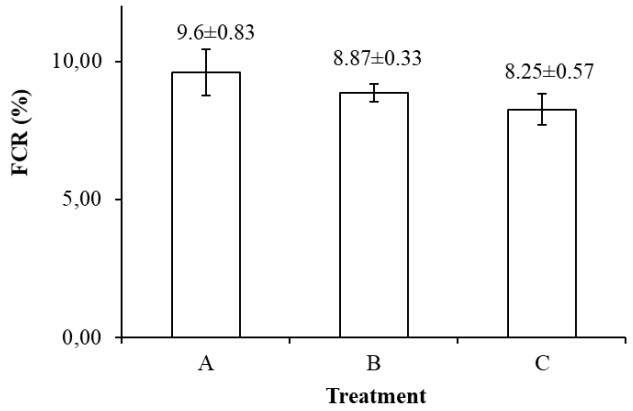

Figure 2. FCR Rates of *Oreochromis niloticus* seeds given artificial feed with different amounts of bacterial content for 35 days of treatment, for each treatment (A, B, C).

Protein Efficiency Ratio (PER). The PER results of *O. niloticus* seeds given artificial feed with different amounts of bacterial content can be seen in Figure 3.

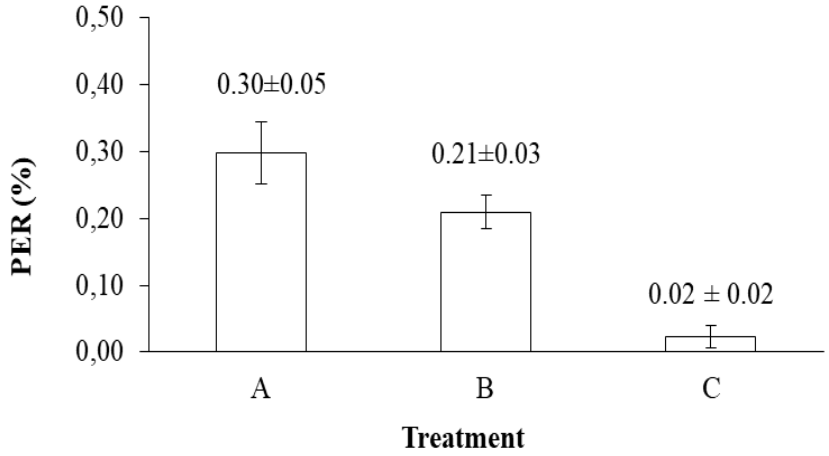

Figure 3. PER rates of *Oreochromis niloticus* seeds given artificial feed with different amounts of bacterial content for 35 days of treatment, for each treatment (A, B, C).

Feed Utilization Efficiency (FUE).The EPP results of *O. niloticus* seeds given artificial feed with different amounts of bacterial content can be seen in Figure 4.

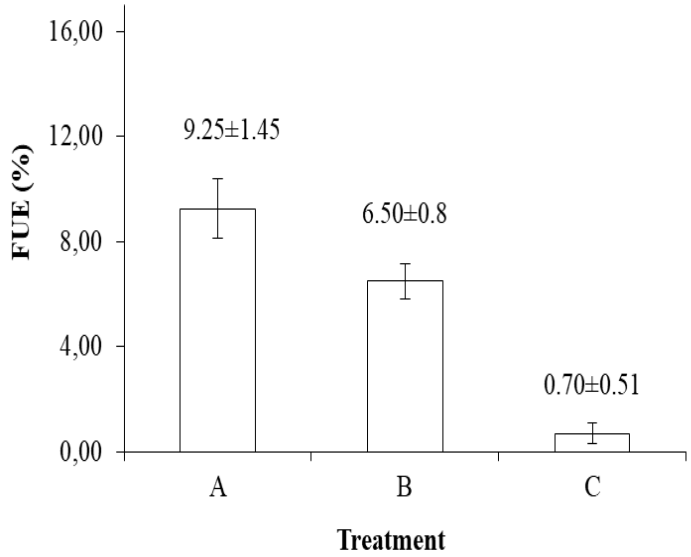

Figure 4. FUE rates of *Oreochromis niloticus* seeds given artificial feed with different amounts of bacterial content for 35 days of treatment, for each treatment (A, B, C).

The mix of fish feed with a consortium of bacteria from the stomach of sea cucumbers showed different protein concentrations. The protein content of the commercial fish feed was higher (31%) compared to the mixed feed containing bacteria. This happened because the commercial feed composition was fish meal, while the consortium mixed feed contained wood bran, so that the crude fiber content was higher in the bacterial consortium (27.62%) compared to commercial fish feed (8%). Meanwhile, non-nitrogenous organic matter (NNOM), nitrogen free extract (NFE) and carbohydrates with a higher concentration (37.64%) were found in the consortium of bacteria from the stomach contents of sea cucumbers compared to the commercial fish feed (31%) as shown in Table 1.

Table 1

Proximate content of commercial fish feed and bacterial consortium

Content (%)	*Commercial fish feed*	*Bacterial consortium from the sea cucumber stomach*
Protein	31	6.43
Crude fiber	8	27.62
Fat	5	2.21
Ash	13	16.30
Water content	12	9.8
NNOM, Carbohydrates, NFE	31	37.64

NNOM-Non-nitrogenous organic matter; NFE-nitrogen-free extract.

Water quality. Results of the measurement of environmental parameters in the tilapia test for feed showed that the salinity and temperature rates tended to be constant for 35 days on the test medium. The salinity during the study on the test medium was about 8 to 10 ppt, while the temperature values ranged from 23.50 to 25.00°C. Furthermore, the pH in the tilapia test container was about 7 to 7.98.

Bacterial density analysis. Bacteria in the water medium (total plate count) before and after feeding the fish specimens (with food mixed with the bacterial consortium, at the tested concentrations) systematically decreased in density for all treatments (Table 2).

Table 2

The number of bacteria (CFU mL^{-1}) in fish medium

Code	*Initial average number of bacteria (CFU mL^{-1})*	*Final average number of bacteria (CFU mL^{-1})*	*Difference in the number of bacteria (CFU mL^{-1})*
A	6.147	4.160	1.987
B	4.853	3.960	893
C	6.160	4.093	2.067

A:25%; B:50%; C:75%.

The difference in the number of bacteria between the beginning and the end of feeding was 1,987 (CFU mL^{-1}) for the treatment A, 893 (CFU mL^{-1}) for the treatment B and 2,067 (CFU mL^{-1}) for the treatment C.

The bioactive compound of the bacterial consortium. The results of the analysis of the active compound bacterial consortium in the stomach contents of the sea cucumbers using the Gas Chromatography-Mass Spectrometer (GC-MS) method showed that the dominant compound (80%) was identified and thus included in the appropriate compound class, with the same molecular weight. The only difference was in the branching of the ester chain. These spectra were thought to be phthalate compounds (e.g. DNOP, di-n-octyl phthalate) (Figure 5), although the molecular ion peak of the mass to charge ratio (m/z) was not visible. The base peak at m/z 149 came from $C_8H_5O_3^+$, which is a typical peak of phthalate compounds with an alkyl group of more than 2 C in its composition, determines the Hβ atom to form a protonated anhydrous ion. The peak at m/z 279 came from $C_{16}H_{23}O_4^+$, due to the release of C_8H_{15} from the molecular ion. The peak at m/z 167 came from $C_8H_7O_4^+$, due to the release of C_8H_{16} from $C_{16}H_{23}O_4^+$. The peak at m/z 113 came from $C_8H_{17}^+$, due to the release of $C_{18}H_{21}O$ from the molecular ion, which then released C_2H_4 to form $C_6H_{13}^+$, followed by the release of CH_2, which appeared at m/z 57, indicating the presence of $C_4H_9^+$, as seen in Figure 5.

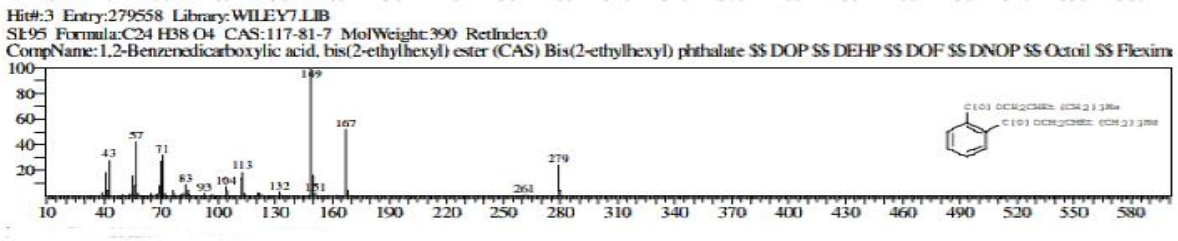

Figure 5. Consortium of bacteria from the stomach contents of sea cucumbers from GCMS.

Among all treatments, the sea bacterial consortium showed that the RGR, TKP, PER, and EPP were always the highest in the treatment A. This means that the lower concentration of bacteria in the consortium, the better the results. The effect of the mix of feed with the bacterial consortium on the bacterial growth in the medium determined a low growth of the tilapia seeds, due to the insufficient availability of feed nutrients. Besides, the differences in the aroma and taste of the different feeds can affect the appetite of tilapia seeds.

The results of this study proved that the sea cucumbers stomach's bacterial consortium in low concentration has the potential to be anti-bacterial agents so that they can improve the water quality conditions and maintain the fish body in optimal conditions, at high survival rates. If the concentration is high, the pellet mixture of the bacterial consortium will be toxic.

The consortium of bacteria from the stomach content of sea cucumbers consisted of 4 types of bacteria, namely *B. toyonensis* strain BCT-7112, *B. aquimaris* strain TF-12, *B. maritimus* strain KS 16-9 and *V. chiguensis* strain NTU-101. The four types of bacteria were synergistic and each of the four types of bacteria were proven to have antibacterial activity against pathogenic bacteria such as *Bacillus cereus* and *Pseudomonas aeruginosa* (Girsang et al 2020), which causes the number of bacteria in the medium fed by the bacterial consortium to decrease, even in the presence of feed residues. Usually, the feed decreases the quality of the medium water and can even infect the fish with bacteria, which eventually cause the death of the domestic fish.

Bacteria contained in the stomach of sea cucumbers were found to contain phthalate compounds. Phthalates are chemical compounds that have an ester functional group attached to a benzene ring. However, phthalate is a chemical compound/substance commonly used as a plasticizer, intending to provide flexibility and durability to polymers, such as Polyvinyl Chloride (PVC). Phthalate ester is a dialkyl or alkyl aryl of phthalic acid (it can be called 1,2-benzene dicarboxylic acid). In their pure state, phthalates are usually clear liquids, some with a pleasant aroma and a faint yellow color. Phthalates usually contain plastic materials that are widely used in everyday's life such as pacifiers or teethers for children. The compounds most used are 2-Ethylhexyl Phthalate (DEHP), diisodecyl phthalate (DIDP) and diisononyl phthalate (DINP). DEHP is the dominant plasticizer used in polyvinyl chloride (PVC) because of its low cost (Xie et al 2016).

The phthalate compounds found in the bacterial consortium lower the number of bacteria after the medium is fed, due to their antibacterial properties (Pringgenies et al 2019). The bacterial consortium added to the fish feed increases the content of non-nitrogenous organic matter, nitrogen-free extract and carbohydrates. Hence, the consortium of sea cucumber gut bacteria is very suitable when used as a mixture of fish feed, but only with the right concentration, because it will be toxic if the concentration is in excess. As elaborated by Shobi & Viswanathan (2018), the di-butyl phthalates have a proven potential as antibacterial and anticancer agents. Furthermore, it was reported that the Di-(2-Ethylhexyl) phthalate confirmed by spectroscopic analysis (IR, HRTOFMS and NMR) showed antibacterial and antifungal activity (Habib & Karim 2009). Moreover, it was reported that the 2-Ethylhexyl Phthalate (DEHP) compound showed antifungal activity against the fungus *Candida albicans* and also had an anti-bacterial activity against the Gram-positive *Sarcina lutea* bacteria. DEHP compounds also have cytotoxic activity against several carcinoma cell lines (Lotfy et al 2018).

Conclusions. The results of this study showed that the proximate content of the consortium of bacteria in the guts of the sea cucumbers is rich in crude fiber (27.62), carbohydrates, non-nitrogenous organic matter and nitrogen-free extract (37.64). The lower the concentration of the bacterial consortium (25%), the higher the fish growth rate. The consortium of bacteria in the stomach content of the sea cucumbers can reduce the number of bacteria (CFU mL^{-1}) in the fish medium. The consortium of bacteria in the stomach contents of sea cucumbers was reported to contain phthalates.

Conflict of interest. The authors declare no conflict of interest.

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