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Potential of probiotics *Bacillus subtilis* to reduce ammonia levels, *Vibrio sp* abundance, and increased production performance of Seaworm (*Nereis sp*) under laboratory scale

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Abstract

This study aims to determine the potential of *Bacillus subtilis* probiotic in reducing levels of ammonia, *Vibrio* sp, and increased production performance in seaworm cultivation (*Nereis* sp.) under laboratory scale. Observation of the performance of seaworms (*Nereis* sp.) was carried out every 10 days which included weight gain (gr), length (cm), total biomass (gr), total bacteria (CFU/mL), total Vibrio sp. (CFU/mL), and total *Bacillus subtilis* (CFU/mL). Water quality measurements include temperature, dissolved oxygen (DO), pH, ammonia, and total organic matter (TOM). Application of probiotics *Bacillus subtilis* has the potential to reduce ammonia concentration, increase growth, and reduce the abundance of *Vibrio* sp under laboratory-scale seaworm cultivation. P3 treatment (0.01 mL with a probiotic density of 10^6 CFU/mL) gave the best results by being able to reduce the ammonia concentration by 47.5%. In summary, the probiotic application using the bacteria *Bacillus subtilis* with different densities able to provide good results in supporting production performance, maintaining the abundance of *Vibrio* sp., and reducing ammonia concentration in seaworm cultivation. This is the first study to report the performance of seaworm production using probiotic agent, research is still needed to determine the digestive enzyme activity of seaworms given probiotics.

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Introduction

Polychaeta is one of the natural foods used in hatcheries as shrimp broodstock besides squid, shellfish, and commercial feed. The nutritional content of Polychaeta is higher when compared to other natural food sources so that Polychaeta worms are widely used as natural food for broodstock. Seaworms (*Nereis* sp.) is one type of Polychaeta worms which contain 56.29% protein, and 11.32% fat (1). The steroid hormones contained in this type of seaworm play an important role in the reproduction of shrimp broodstock (2). The use of seaworm as shrimp feed, has been widely researched, especially in the process of ripening gonads and spawning of broodstock. Previous research reported the success of laboratory-scale *Dendronereis pinnaticirris* hatchery which has produced larvae up to young worms that are ready to be used as natural shrimp feed (3). The availability of natural food in cultivation activities is very important, therefore seaworms cultivation needs to be done to provide natural food and efforts to maintain availability in nature. Cultivation of scrambled worms has not developed much in Indonesia due to limited research on aspects of biology, ecology, reproduction, and aspects of mass cultivation as well as economic aspects. The problem that also arises in Polychaeta cultivation is the high ammonia content in maintenance caused by the accumulation of organic material from the feed and metabolism, the inorganic nitrogen compounds produced are in the form of NH_3-N and NH_4+ which are toxic compounds for aquatic biota (4). The use of probiotics as a biological approach to water quality

management by utilizing bacterial activity in remodeling organic matter in cultivation systems. The bioremediation agent must have the metabolic ability for oxidation, ammonification, nitrification, denitrification, sulfurization, and nitrogen fixation reactions so that it can directly overhaul organic and hazardous materials in the aquaculture systems (5). On the other hand, the use of natural-derived Polychaeta for cultivation purposes has decreased for biosecurity reasons (6). Polychaeta is benthic microfauna in shrimp and sediment ponds found in coastal areas. Polychaeta collected from nature may carry pathogens as well as viruses in the digestive tract by consuming sediment (7). Sediment can act as a habitat, vector and growth of pathogens such as *Vibrio* sp. and WSSV (*White Spot Syndrome Virus*) (8) therefore Polychaeta can act as an infectious host if given as natural food. The spread of *Vibrio* sp. in shrimp through several ways, namely through the feed, wounds on the body surface, and gills that cause bacteria to pass through the epithelial tissue and form colonies. Polychaeta plays a role in the spread of *Acute Hepatopancreas Necrosis Disease* (AHPND) which is caused by *Vibrio parahaemolyticus* and *Hepatopancreatic Microsporidiosis* caused by *Microsporidian Enterocytozoon hepatopenaei* (EHP) microspores (9). Probiotics can be used as a disease biocontrol agent in aquaculture and as an effort to reduce the antibiotic application (10). *Bacillus* sp. are lactic acid bacteria which can act as antagonistic bacteria to pathogens and reduction of *Vibrio* sp. in shrimp farming. Probiotics containing *Bacillus* sp. also aimed to improve growth performance in aquaculture. According to reports from several studies stated that *Bacillus* sp. can be widely used as microorganisms in probiotics to increase survival, growth and stimulate immunity in shellfish aquaculture.

Based on this, the purpose of this study was to determine the potential of probiotics *Bacillus subtilis* in reducing levels of ammonia, *Vibrio* sp, and production performance of seaworms (*Nereis* sp.) on a laboratory scale.

Materials and methods

Time and research location

This research was conducted from January to April 2020 at the Center for Brackish Water Fisheries. The use of premises, tools, and various facilities has obtained permission from the parties concerned and has followed various Standard Operating Procedures (SOP). The use of seaworm test animals (*Nereis* sp.) is intended only for laboratory-scale research.

Research design

The research design used a completely randomized design (CRD) with 4 treatments and 3 replicates. The probiotic used was pure isolate *Bacillus subtilis* with the addition of 10 ml of Molasses/treatment as a nutrient for bacteria. Treatment were as follows: P1: with the bacterial density of 10^{10} was given as much as 50 ml. P2: with the bacterial density of 10^{8} was given as much as 10 ml. P3: with the bacterial density of 10^{6} was given as much as 0.01 ml. D: control (without probiotic *Bacillus subtilis*). Plastic-based seaworm rearing container with one drain outlet on the front side of the container with a diameter of 2 cm. The container used has dimensions L×W×H (60 cm × 30 cm × 25 cm) with a maximum volume of 45 liters. Aeration is given as much as 2 pieces in each container used. Before use, each container was cleaned using water containing 10 mg/l chlorine. The water medium is stored in a round container with a diameter of 1.5 m, a height of 1.5 m, and a maximum volume of 2.64 m^3 and then sterilized using chlorine 25 mg/l (Figure 1).

Figure 1: The seworms (*Nereis* sp.) rearing container used in this study.

Substrate preparation and maintenance medium for seaworm

The source of maintenance water comes directly from the sea which has been stored in the main reservoir with salinity ranging from 28-30 ppt. Water to be used during maintenance requires a low salinity ranging from 18-22 ppt, dilution is required until the salinity is in the range. Water needs as much as 20 liters/container. The substrate used as a medium for seaworms is in the form of live fine sludge obtained from seaworm cultivators. The substrate was then dried and weighed as much as 25 kg/container. The ready substrate is soaked in seawater in each container for 7 days so that the substrate texture is softer. The substrate height is 10 cm and the water level is 3 cm calculated from the substrate surface. Water changes are carried out every 10 days with a percentage of 40% (3).

Preparation of seaworm (*Nereis* sp.)

Seaworms (*Nereis* sp.) are obtained through collectors in living conditions with a weight ranging from 0.69 g - 1.26 g and a length of 5.6 - 14.5 cm with a stocking density of 40 individuals/container. Worms are selected according to the criteria, namely: intact and unbroken limbs. The experimental animals were acclimatized for two weeks in a plastic box measuring L × W × H (60 cm × 30 cm × 25 cm) which already contained fine mud and seawater substrate (3), salinity 28-30 ppt, and equipped with aerator equipment to meet the oxygen needs of the worm. Maintenance is carried out for 30 days in a controlled room. The feed used is commercial feed with a protein content of 48%, 9% fat, and 2.5% fiber. The dose of feed given is 3% of the worm biomass. The frequency of feeding was twice, carried out at

06.00 am and 17.00 pm, the dead worms were removed and recorded for biomass and population calculations.

Isolation of probiotic bacteria

The pure isolate of *Bacillus subtilis* obtained from the intestines of milkfish (*Chanos chanos*). Each sample of 1 g was suspended in 3 ml of NaCl solution (Sigma-Aldrich, US) 0.9%, then put into a test tube containing 9 ml of Tryptic Soy Broth (TSB) (Oxoid, Canada) and incubated for 24 hours at 30°C, followed by incubation at 45°C for 10 minutes in the oven to activate the sporulation process. Ethanol 50% (Sigma-Aldrich, US) was added as much as 20 ml to each sample then incubated again at a lower temperature of 20°C for 1 hour, then centrifuged at 10.000 rpm. The supernatant produced was poured and incubated at 105°C in an oven for 5 minutes. Dry pellets were dissolved in 20 ml of sterile physiological saline and diluted serially to 10^{-4} in 10^{-1} increments. A total of 0.1 ml from each dilution series was spread in a Petri dish (Fisher Scientific, US) containing Tryptone Soya Agar (TSA) (Oxoid, Canada) which had been added with Polymyxin B antibiotic (Oxoid, Canada) 5 mg/L. The culture was then incubated for 24 hours at 30°C. Colonies isolated from the plates were purified and then Gram and spore stained were carried out for the further selection of bacteria in basal media.

Sample analysis

Measurement of temperature and Dissolved Oxygen (DO) using a DO meter (Krisbow KW0600752), pH using a pH meter (HANA HI 98107), measurements are carried out every day at 06.00 am. Ammonia and Total Organic Matter (TOM) are analyzed every 10 days at the Water Quality Laboratory of BBPBAP Jepara, Central Java, Indonesia, total bacteria (CFU/ml), abundance *Vibrio* sp. (CFU/ml), and total *Bacillus subtilis* (CFU/ml).

Measurement of seaworm growth is done every 10 days which includes: weight gain (gr), length (cm), total biomass (gr), total bacteria (CFU/ml), abundance *Vibrio* sp. (CFU/ml) and total *Bacillus* sp. (CFU/ml) in seaworms. The number of samples used in the measurement of growth was 5 individuals in each research container. Substrate excavation was carried out to obtain seaworm samples carried out at 5 different points, from corner and in the middle of the rearing container. Measurement of length and weight was carried out quickly, 3 seaworms from 5 samples in each of the measured study containers were then placed on a sterile Petri dish for intestinal collection. Seaworm samples were dissected using a sterilized dissecting set. Intestinal retrieval is carried out carefully.

Production performance

Production performance parameters for seaworms are determined according to the following: Standard length growth was observed every 10 days using Apriani *et al.* (11). The specific growth rate (SGR%) was calculated every 10 days using Schulz *et al.* (1), Survival Rate (SR%) was observed at the end of maintenance using the following Wiradana *et al.* (8), and the Biomass is the amount of animal biomass added over a certain period of time

Data analysis

The effect of each treatment on predetermined parameters has been tested with single-factor ANOVA at the 95% confidence level. Duncan test to determine the best treatment. The software used is IBM SPSS version 25.0.

Results

Water quality

Water quality data including temperature, pH, DO, ammonia, TOM can be seen in Table 1. Temperature, pH, and DO in each treatment and control showed high values so that the resulting value was not significant ($P>0.05$). The highest ammonia value was found at P1 ($P<0.05$), indicating a significant difference with P3 but not significant with P2 and control ($P>0.05$). Based on the results of statistical tests on the TOM parameter, the highest value was found in P1 which in contrast showed a significant difference with control ($P<0.05$), while the P2 and P3 treatments did not show a significant value ($P<0.05$).

Total water bacteria

Data on total bacteria and total *Bacillus subtilis* in each treatment on seaworm rearing media can be seen in Table 2. The total bacteria at P1, P2, and P3 showed insignificant differences ($P>0.05$) but all treatments when compared to controls. The predominant bacteria are the bacterium *Bacillus subtilis*. It shows that P3 is significantly different from control ($P<0.05$), while P3 is not significantly different from the other two treatments.

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Total gut bacteria

Data on total bacteria count, *Bacillus subtilis*, and *Vibrio* sp. in each treatment in the intestines of seaworms can be seen in Table 3. The highest value on P1 and the lowest on Control showed insignificant differences ($P>0.05$). The values contained in P1, P2, and P3 are not significant difference ($P>0.05$). When compared, the total value of bacteria at P1 and P3 showed a significant difference ($P<0.05$). Although the total bacteria in P3 was low when compared to P1 and P2, the total *Bacillus subtilis* which was the dominant bacteria with a high value was found in P3,

there was a significant difference between all treatments (P<0.05). Total *Vibrio* sp. at P1 was higher than the other treatments, with a total of *Vibrio* sp. lowest at P3 (P<0.05) when compared to other treatments.

Production performance parameters

Production performance observations including final weight (Wt), Specific Growth Rate (SGR), standard length (cm), survival rate (SR), and biomass (g) can be seen in table 4. The Wt, shows that there is no significant difference (P>0.05) from the three treatments to the control. The highest SGR and Biomass values were found in P3 which showed a significant difference (P<0.05) when compared to the control and there was no significant difference between P1 and P2. The results of observations on the SR parameters and the standard length gain, P3 showed high values of all treatments. When compared, the P3 value to the control shows a significant difference (P<0.05), while it does not show a significant difference to other treatments.

Table 1: Physicochemical parameters of water

Parameters	Treatments			
	P1	P2	P3	Control
Temperature (°C)	28.73±0.40^{a}	28.26±0.08^{a}	28.13±0.12^{a}	28.46±0.08^{a}
pH	6.5±0.05^{a}	6.4±0.08^{a}	6.4±0.05^{a}	6.5±0.05^{a}
DO (mg/L)	5.23±0.17^{a}	5.56±0.17^{a}	5.26±0.12^{a}	5.30±0.05^{a}
Ammonia (mg/L)	0.60±0.10^{b}	0.35±0.05^{ab}	0.31±0.06^{a}	0.39±0.05^{ab}
TOM (mg/L)	119.23±3.79^{b}	109.66±2.73^{ab}	104.66±5.22^{ab}	93.26±7.59^{a}

*TOM: Total Organic Matter, DO: Dissolved oxygen. Data are displayed using the mean ± SE (standard error). Mean on the same line with different manuscripts showed a significant difference (P<0.05).

Table 2: Total bacteria count and *Bacillus subtilis* count

Parameters	Treatments			
	P1	P2	P3	Control
Total Bacteria count × 10^5 (CFU/ml)	4.18±0.26^{b}	4.43±0.15^{b}	4.28±0.28^{b}	2.72±0.19^{a}
Total *Bacillus subtilis* × 10^3 (CFU/ml)	3.66±0.83^{ab}	5.11±0.77^{ab}	5.89±0.67^{b}	3.00±0.38^{a}

Data are displayed using the mean ± SE (standard error). Mean on the same line with different manuscripts showed a significant difference (P<0.05).

Table 3: Total Bacteria, Total *Bacillus subtilis,* and Total *Vibrio* sp. in the intestine of seaworms

Parameters	Treatments			
	P1	P2	P3	Control
Total Bacteria Count × 10^6 (CFU/ml)	5.74±067^{ab}	5.49±0.56^{ab}	4.12±0.26^{a}	3.72±0.76^{b}
Total *Bacillus subtilis* × 10^4 (CFU/ml)	2.55±0.67^{a}	2.77±0.77^{a}	4.80±0.56^{b}	2.23±0.29^{a}
Total *Vibrio* sp. × 10^3 (CFU/ml)	3.82±0.62^{b}	2.90±0.35^{ab}	2.00±0.41^{a}	3.54±0.41^{ab}

Data are displayed using the mean ± SE (standard error). Mean on the same line with different manuscripts showed a significant difference (P<0.05).

Table 4: Production performance of seaworms after administration of the probiotic *Bacillus subtilis*

Parameters	Treatments			
	P1	P2	P3	Control
W_t (g)	1.0±0.28^{a}	1.26±0.10^{b}	1.29±0.06^{b}	1.08±0.14^{ab}
SGR (%)	0.26±0.37^{ab}	0.86±0.06^{ab}	1.01±0.29^{b}	0.14±0.05^{a}
SR (%)	75.89±3.09^{a}	75.33±2.54^{a}	86.66±4.44^{b}	72.00±1.16^{a}
Standard length (cm)	1.53±0.29^{b}	2.03±0.16^{b}	3.07±0.34^{c}	0.66±0.10^{a}
Biomass (g)	29.93±2.80^{ab}	31.80±2.48^{ab}	37.00±3.71^{b}	25.38±1.51^{a}

Data are displayed using the mean ± SE (standard error). Mean on the same line with different manuscripts showed a significant difference (P<0.05). W_t: Weight total, SGR: Specific Growth Ratio, SR: Survival Rate.

Discussion

Water quality

The temperature and pH values in the maintenance media have been classified in the optimum range for seaworm cultivation. This is following previous research which states that the optimum temperature is 28-30.5°C, the optimum value is pH 6-7 (1). Each treatment has a DO value ranging from 5.23 - 5.56 mg /l. This value is not included in the optimum range based on previous research, which is 6-8 mg /l (2). However, this can still be tolerated by seaworms because, in natural habitats, seaworms can live in the DO range between 1.01-8.0 mg/l. Ammonia concentration and high temperature in the sludge medium were the causes of the decrease in DO in this study, which in turn was able to reduce growth and increase the mortality of experimental animals.

Based on TOM observations, at P1, P2 and P3 have values above the optimal average ranging from 55-95 mg/l. The addition of molasses as a source of carbon and nitrogen affects the TOM value due to the addition of organic matter resulting in a buildup of organic matter such as remaining feed and animal waste. The decomposition of organic matter by heterotrophic bacteria on P1 takes place more slowly. In addition, seaworms are deposit feeders that consume sediment deposits, allowing them to use organic matter contained in sediments directly. Digestible organic matter consists of only <1% based on the total sediment consumed (14).

Bacillus sp. a type of bacteria that can grow in various conditions and is saprophytic and can form endospores that remain active in the digestive tract (15). The endospores produced will remain active for a long period, so they are suitable for use as probiotic candidates. Ammonia value in each treatment exceeds the tolerance limit for aquatic biota, namely 0.01 mg/l. High ammonia values can be caused by high density, namely 33 individuals/containers and high total organic matter (TOM). The ammonia concentration in P3 treatment is lower when compared to P1. This is due to the density of *Bacillus subtilis* different in each treatment which resulted in the performance of probiotics not optimal in degrading organic matter. The P3 treatment was able to reduce the ammonia concentration by 47.5% compared to the P1 treatment which was 37.8%. This proves that the probiotic bacteria used can utilize inorganic nitrogen for cell growth and multiplication. According to Kim *et al.* (16), as much as 12.4% nitrogen contained in ammonium will be used for the addition of bacterial biomass. Furthermore, Ebeling *et al.* (17) stated that the ammonia that has been formed will be used by heterotrophic bacteria and synthesized into protein due to the presence of organic carbon compounds (such as sugar and molasses). *Bacillus subtilis* is a type of heterotrophic bacteria that will utilize N either in organic or inorganic form for the formation of bacterial biomass so that the concentration of N in water can be reduced.

In addition, water quality parameters such as temperature and pH have an important influence on the rate of ammonia concentration, namely the higher the pH and temperature, the ammonia levels and their toxicity tend to increase as found in the P1 treatment in this study. This is because at high temperatures and pH more nitrogen will be formed in the form of NH_3 which is part of TAN.

Abundance *Vibrio* sp. in sea worms with the use of the probiotic *Bacillus subtilis*

Abundance *Vibrio* sp. shows a significant difference ($P<0.05$) in treatment P1 and P3. Treatment with probiotics showed that total *Bacillus subtilis* in treatment P3 could reduce the abundance of *Vibrio* sp by 75.90% compared to treatment P1. Abundance of *Vibrio* sp. can be influenced by the presence of the *Bacillus subtilis* which suppresses the growth of *Vibrio* sp. by producing metabolites in the digestive tract. *Bacillus subtilis* is used as a probiotic product in aquatic-terrestrial animals which will be active while in the digestive tract (18). Abundance *Vibrio* sp. in all treatments was 10^3 which is still low while the infection threshold for *Vibrio* sp. is 10^4 and likely will not cause vibriosis if seaworms are used as natural feed for shrimp broodstock (19). It can be explained that the *Vibrio* sp. depends on density and dominance in an environment and occurs naturally in all aquatic environments and is mostly found in shrimp farming.

Production performance

Based on the results of the study, it can be seen that the value of the final weight, SGR, SR, length, and biomass growth standard. In seaworms with the highest value is found in the P3 treatment when compared to other treatments. This can be caused by the use of the *Bacillus subtilis* bacteria as a probiotic agent capable of influencing the growth of seaworms. The probiotics given enter the digestive tract through water and stick to the host. The use of probiotics with *Bacillus subtilis* mixed in feed can stimulate growth in other aquatic biota. Probiotic applications can be in the form of mono strains (as was done in this study), multiple, or even in combination with prebiotics or better known as synbiotics. Encapsulation of probiotics with live feed is a good approach to convey the effectiveness of probiotics to aquatic animals (20). Digestive enzymes can be triggered by giving probiotics which can promote better growth than without probiotics. SGR and biomass values on P1 treatment and control were lower than those of other probiotic treatments. Low SGR can be caused by several reasons, such as feed quality and feeding habits and the health status of aquatic animals which can play an important role in determining SGR (21).

The results of the observation on the SR parameter (%) showed that the P3 treatment had a greater value than all other treatments. The substrate used can affect survival in seaworm maintenance. The finer the substrate is used, the less energy use will be. Apart from the substrate, high

ammonia levels can also affect survival in seaworm maintenance. Ammonia in the form of NH_3 molecules can penetrate cell membranes faster and affect the physiology of organisms (22-24).

Conclusion

Probiotic application using the bacterial agent *Bacillus subtilis* with different densities able to provide a significant difference in the growth of seaworms. Apart from increasing growth, *Bacillus subtilis* as a probiotic agent is also able to reduce the number of pathogenic bacteria in the intestine. During this study, the best treatment was P3 treatment, namely probiotics with a density of 10^6 bacteria to increase growth and reduce the pathogen *Vibrio* sp. Further research is needed to determine the activity of enzymes that occur in the intestines of marine worms in the application of probiotics, prebiotics, synbiotics and immunostimulants.

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Competing interests

The authors declare that they have no competing interests.

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إمكانية المعزز الحيوي Bacillus subtilis في تقليل مستويات الأمونيا وتوافر انواع بكتيريا الضمة وزيادة الأداء الإنتاج لدودة البحر مختبريا

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الخلاصة

تهدف هذه الدراسة إلى إمكانية المعزز الحيوي Bacillus subtilis في تقليل مستويات الأمونيا وبكتيريا الضمة (vibrio) مع زيادة الأداء الإنتاجي في استزراع دودة البحر (Nereis sp) مختبريا. تمت مراقبة الأداء الانتاجي لدودة البحر كل ١٠ أيام والذي تضمن الزيادة الوزنية (غرام) والطول (سم) والكتلة الحيوية الكلية (غرام) والعدد الكلي للبكتيريا (وحدة تشكيل المستعمرات/ مل) والعدد الكلي لبكتيريا الضمة (وحدة تشكيل المستعمرة/ مل) والعدد الكلي لل Bacillus subtilis (وحدة تشكيل المستعمرة/ مل). شملت قياسات جودة المياه كل من درجة الحرارة والأوكسجين المذاب ودرجة الحموضة والأمونيا والمواد العضوية الكلية. إظهرت النتائج ان استخدام المعزز الحيوي Bacillus subtilis لديه القدرة على تقليل تركيز الأمونيا وزيادة النمو والتقليل من وفرة بكتيريا الضمة (vibrio) في ظل استزراع الدودة البحرية مختبريا. أعطت المعاملةP3 (٠,٠١ مل مع كثافة المعزز الحيزي ١٠٦ وحدة تشكيل المستعمرة/ مل) أفضل النتائج من خلال قدرة المعزز الحيوي على تقليل تركيز الأمونيا بنسبة ٤٧,٥٪، ومن هنا نستنتج بأن تطبيق استخدام المعززالحيوي Bacillus subtilis بكثافات مختلفة قادرة على اعطاء نتائج جيدة في دعم الأداء الإنتاجي والحفاظ على وفرة بكتريا الضمة(vibrio) وتقليل تركيز الأمونيا في استزراع دودة البحر، وهذه هي الدراسة الأولى التي تبحث عن الأداء الإنتاجي لدودة البحر باستخدام عامل المعزز الحيوي، ولا تزال هناك حاجة للبحث وذلك لتحديد النشاط الإنزيمي الهضمي للديدان البحرية المعاملة بالمعزز الحيوي.