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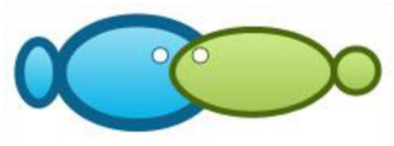

Explorations of symbiotic microbe from sea cucumber gut as an anti-multi-drug resistant microbe agent for utilization in hand sanitizer products

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Introduction. Sea cucumber is one of the medicinal foods for health (Pringgenies et al 2018). Sea cucumbers (Phylum Echinodermata), are marine invertebrates commonly found in shallow waters. In Asia, people use this animal as medicines (Abraham et al 2002; Dang et al 2007; Farouk et al 2007; Althunibat et al 2009).

Sea cucumbers found in Bandengan waters (Jepara, Indonesia) contain saponins (Martoyo et al 2006). This steroidal sapogenin content is known to have antibacterial effects on gram positive and gram-negative bacteria. Besides saponins, it was reported that pure sea cucumber extract produced holotoxins which had the same effect as antimycin. Antimicrobial sensitivity test on samples of crude extract of brown sandfish (*Bohadschia marmorata*) and leopard sea cucumber (*Bohadschia argus*) showed the ability to inhibit the growth of tested bacteria *Staphylococcus aureus, Escherichia coli, Vibrio anguila, V. vionovica, Bacillus subtilis, Pseudomonas* sp. (Pinggenies 2014). Samples of crude extract of *Sticophus variegatus* was shown to inhibit the growth of *Escherichia coli*, *Pseudomonas* sp., *V. vionovica* (Pringgenies et al 2018). The crude extract of greenfish sea cucumber (*Sticophus chloronotus*) and golden gamat (*Sticophus herrmanni*) has the ability to inhibit the *S. aureus, E. coli, V. anguila, V. vionovica.* Furthermore, *S. herrmanni* extract has antibacterial potency against periodontopathogens (Weisburg et al 1991).

Based on above findings, the symbiont bacteria found on the sea cucumber digestive organ are important to be examined as anti-bacterial agents. The purpose of the study is to determine the potency of symbiont bacteria from the sea cucumber stomach as an antibacterial against MDR pathogens. This product is aimed as hand sanitizer products, specifically.

Material and Method

Sampling. Specimen of fresh sea cucumber *Holothuria atra* and *Holothuria leucospilota* were collected from Bandengan waters, Jepara, Indonesia. The samples were kept in polyethylene plastic bags (Whir-pak, Nasco, USA) and placed in a coolbox.

Isolation. Samples of sea cucumber were dissected to get its stomach content. One gram of material was placed in 9 mL sterile sea water and diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. From each dilution, 100 µL was transferred into microbial Petri dish and incubated for 2 x 24 hours at room temperature. Purification of bacterial isolates was carried out by the streak method until pure culture was obtained.

Antimicrobial activity screening. Determination of bacterial isolated from gut of sea cucumber was using the overlay method. The 6-8 isolates were placed on a petri dish with agar medium. The petri dish was incubated for 4 days at room temperature. One percent of culture (v/v) of each target MDR bacteria in the logarithm phase (ca. 10^9 cells. mL^{-1}) was mixed very gently then poured on inoculated isolate samples media. The cultures were then incubated at room temperature for 48 hours. Anti-bacterial activity was determined by the presence of barrier zone formation around the isolates.

Isolation of pathogenic bacteria (MDR). Various clinical specimens (blood, feces, urine etc.) were obtained from the Laboratory of Clinical Microbiology, Faculty of Medicine, Diponegoro University, Semarang. All speciments were cultured on Nutrient Agar, Mac Conkey and Blood agar, CHROM agar *S. aureus* and CHROM agar MRSA (ITK Diagnostic). After 18-24 hours of incubation at 37°C, identification of bactery was carried out biochemically tests, namely: bacterial colony morphology, catalase test, coagulase test, gram stain, haemolysis in blood agar, pink colony in CHROM agar *S. aureus* and CHROM agar MRSA media (grampositive bacteria), Mac Conkey agar plate (gram negative bacteria).

Inoculum preparation. Isolation of bacteria was suspended in sterile NaCl water at concentration of $<300.10^6$ mL^{-1} to get 0.5 McFarland. The bacterial suspension was then directly used for the diffusion test.

Antibacterial sensitivity test. Disk diffusion method was employed with reference to CLSI (Clinical Laboratory Standards Institute / NCCLS 2005). Briefly, bacterial spacing was spread on Mueller-Hinton (Oxoid) agar surface (Radjasa et al 2001). Antibiotic disks (Oxoid) were placed on surface so as Mueller - Hinton Bacterial incubation can be carried out. After 24 hours incubation at room temperature, the inhibition zone can be measured by means of a caliper. The sensitivity category of bacteria (sensitive, intermediate susceptible or resistant) was determined by comparing the inhibition zone diameter (NCCLS 2005).

Quality control. Sensitivity test results were determined by using MRSA and ESBL strains quality control.

Antimicrobial sensitivity test. Antimicrobial sensitivity tests were carried out using Dish diffusion method (NCCLS 2005). Muller Hinton (MH) agar plate was prepared. Then bacterial suspension was made in sterile NaCl to suit the turbidity standard of 0.5 MacFarland. Bacterial suspensions were spread on the MH agar plate medium with sterile cotton swabs. An antibiotic disk (Amoxicilin) was then placed on each agar surface prior

to incubation at 37°C for 18-24 hours. After incubation, inhibition zone formed on each disk can be measured by means of a caliper. The NCCLS standard was used to categorize the results namely susceptible/sensitive, intermediate, and resistant categories. The control strains used were MRSA and ESBL.

DNA extraction. Selected bacterial isolates were cultured in 50 mL of ZoBell 2216E liquid medium at 20°C for 24 hours. The cultures were then collected by centrifugation and washed prior to suspension in sterile distilled water. DNA extraction was carried out by mixing 40 µL of bacterial suspension, 10 µL of Proteinase K (1 mg mL^{-1}) (Sigma Chemical Co., St. Louis, USA) and 50 µL two fold buffer. The mixture was heated at 60°C for 20 minutes and 100°C for 10 minutes. After rapid cooling in ice for 10 minutes, the mixture was centrifuged for 5 minutes at 8000 rpm (Sabdono et al 2000).

DNA amplification. DNA amplification was carried out by PCR method (Radjasa et al 2001). The primer used was (Forward: 5'-AGAGTTTGATCMTGGCTCAG-3'; positions 8-27 and 1500 Reverse primer: 5'-GGTTACCTTGTTAC GACTT-3'; positions 1510-1492 based on 16S rRNA *E. coli* numbering (Weisburg et al 1991).

DNA amplification by PCR was carried out with DNA thermal cycler (Mini cycler TM, MJ Research Inc., Watertown, MA, USA) with the following temperature treatment: Initial denaturation at 94°C for 2 minutes, then 30 cycles of denaturation (94°C for 2 minutes), annealing (45°C for 2 minutes), and extension (72°C for 2 minutes), as well as the last extension at 72°C for 3 minutes. Electrophoresis was carried out by inserting 1 µL of aliquot of PCR product into a 1% agarose gel, well placed on a 50X TAE buffer, then monitored the DNA amplification.

DNA sequencing. The results of amplification with PCR were purified and concentrated by means of Microcon-100 microconcentrator (Amicon, Beverly, MA, USA), according to the manufacturer's instructions. The 16S rDNA gene sequence reaction was prepared by employing SequiTherm Long-Read Sequencing Kit (Epicentre Technologies, Madison, WI, USA). PCR products were synchronized using 8 primers: 20F, 300R, 520R, 810R, 1100R, 1400R, 1100F and 1340F (Thompson et al 1994).

Bioactivity test crude extract isolates bacteria from sea cucumber. The test was aimed to determine which crude extract had the ability to inhibit the growth of test bacteria (*E. coli* and *S. aureus*). The method used in this bioactivity test was the Kirby-Bauer agar diffusion method. Paper discs were placed on agar media with tested bacteria on it, then dripped with crude extracts (from extracts of non-polar, semi-polar and polar compounds). The bioactive compounds contained in each extract would diffuse on the medium and would affect bacterial growth. Antimicrobial growth inhibition of microorganisms was seen as a clear area around disc paper. The extent of clear area was an indication of the sensitivity of microorganisms to antimicrobial substances or compounds (Lay 1994).

Solubility test. Solubility test was carried out using aquadest and methanol. The test was aimed to determine the solubility of the crude extract against the solvent used. 1.5 mL of methanol was poured into a test tube, then 1.0 mg crude extract were mixed in a solvent. The crude extract was considered as soluble if the mixture became homogeneous (Radjasa 2001).

Bacterial sensitivity test for solvent. Sensitivity test was aimed to determine the effect of solvents on bacterial sensitivity so that the presence of anti-bacterial potential caused by solvents can be avoided.

Determination of levels (Minimum Inhibitory Concentration). This test aimed to determine the appropriate levels to be used as a reference for the formula for making antiseptic gel preparations.

Antiseptic gel preparation. Gel preparations for sea cucumber symbiont bacteria isolates which have potential as anti-bacterial agents were made based on the results of concentration test in the previous stage. Carbopol was developed in hot water, then stirred. Bacterial extracts were mixed with other materials until well-blended, then was placed into the carbopol. Water was added to the mixture to the desired volume, then added TEA drop by drop until a clear gel formed. The antiseptic tests on MRSA and ESBL bacteria were carried out with different concentrations, namely 100, 250, 500 and 1000 µg each disk.

Results and Discussion

Morphology of isolate bacteria. Sea cucumber produced 24 isolates in the gastric contents of *H. leucospilota*, and 18 isolates in *H. atra*. Each bacterium was characterized by its shape, edge, surface, color, and size of the colony. The number of irregular types of bacterial colonies was 24 isolates. The round was represented by 14 isolates, and the rhizoid shape by 4 isolates. Edge types observed as smooth were 22 isolates, rhizoid type were 5 isolates, and irregular type were 15 isolates. The surface shape known to be raised were 11 isolates, growth into media were 2 isolates, convex were 12 isolates, flat were 9 isolates, plate were 5 isolates, and umbonate were 3 isolates. Large sizes of bacterial colonies identified were 12, moderate colonies were 19, and small colonies were 11 isolates. The observed bacterium colors were: yellow 16 isolates, orange 12 isolates, white 9 isolates, and clear 3 isolates (Table 1).

Table 1

Identification of bacterial symbology morphology concerning shape, edge, surface, size, and color

Morphology		*Bacterium code*	
		TB (Isolate)	*TH (Isolate)*
Shape	Irregular	14	10
	Round	7	7
	Rhizoid	3	1
Edge	Smooth	10	12
	Rhizoid	5	ND
	Irregular	9	6
Surface	Raised	5	6
	Growth into media	2	ND
	Convex	8	4
	Flat	4	5
	Plate	4	1
	Umbunate	1	2
Size	Large	7	5
	Moderate	9	10
	Small	7	4
Color	Yellow	12	4
	Orange	7	5
	White	4	5
	Transparent	1	2

ND - not detected.

Test of symbiotic bacterial activity against MDR pathogens. The highest activity against MDR bacteria were isolates of TB (TB.07, TB.12, TB.18) and TH (TH.20, and TH.15). The bacteria were cultured and then were subjected to antagonistic test (Table 2).

Table 2

Symbiotic bacterial activity test results for MDR pathogens

Code	Number of active isolate					
	MRSA			ESBL		
	+	++	+++	+	++	+++
TB	1	2	4	2	5	3
TH	4	n/a	1	1	1	3

(+) = Low activity, (++) = Medium activity, (+++) = High activity.

Antagonistic test. Antagonistic tests showed synergy between TB symbionts TB 07, TB.12, TB.18, TH.15 and TH.20. This was indicated by the absence of a barrier zone around the colony.

Antiseptic test of symbiotic bacteria on MDR pathogenic bacteria MDR. There were 5 bacterial isolates tested, namely isolate TB-7, TB-18, TH-20, TB 12 and TH-15 and test the bacterial consortium of 5 isolates against MDR bacteria as shown in the Table 3 as follows:

Table 3

Results of symbiotic bacterial AST on MDR bacteria

Code	Anti MDR pathogen activity (mm)							
	MRSA (µg per disk)				ESBL (µg per disk)			
	1000	500	250	100	1000	500	250	100
Co-culture	2.8±0.49	1.7±0.6	-	-	-	-	-	-
TB-7	-	-	-	-	-	-	-	-
TB-18	-	-	-	-	-	-	-	-
TH-20	-	-	-	-	-	-	-	-
TB-12	-	-	-	-	-	-	-	-
TH-15	-	-	-	-	-	-	-	-

Co-culture - consortium culture.

The results showed that none of the bacterial isolates had positive activity on the MRSA and ESBL type MDR test bacteria. However the bacterial consortium had positive activity against the bacterium MRSA test at concentrations of 1000 µg each disk (2.8±0.49 mm) and 500 µg per disk (1.7±0.6 mm), respectively.

Molecular philogenetic study. DNA amplification from the 4 sea cucumber symbiotic isolates with the best potential as anti-MDR (active TB.07 & TB.12, TH.15 & TH.20 isolates) were performed using ribosomal primers (F: positions 8 to 27; and 1500 R: position 1510 up to 1492 from numbering 16S rRNA *E. coli*). The results of DNA amplification can be seen in Figure 1.

As can be seen in Figure 1, all isolates produced a single band with a size of about 1,500 bp according to the comparison using DNA markers. This size was in accordance with the size expected from 16S rRNA bacterial genes which were around 1,500-1,600 bp. DNA amplification of anti MDR mollusk isolates with a single band showed that the primer used was a specific primer for amplifying the 16S rRNA gene in bacteria. Similarly, the conditions used in the amplification reaction were the ideal conditions (Table 4).

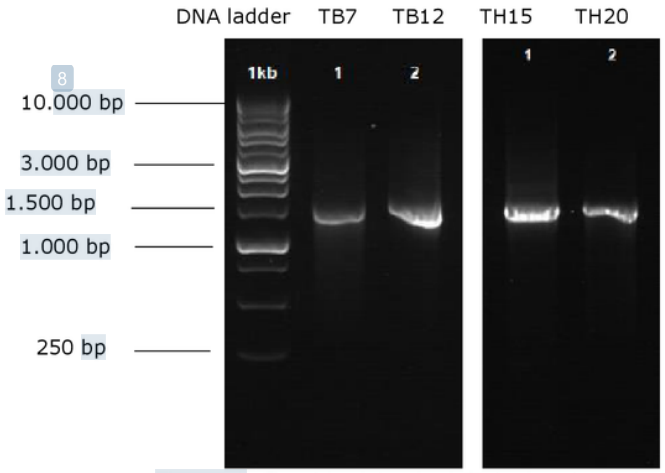

Figure 1. Symbiont bacterial electrophoresis gel.

Table 4

Symbiotic bacteria DNA sequencing results

Isolate code	*DNA sequence*
TB.07	TGCTATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTT AGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATT AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT GAGAGGGTGATACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
TB.12	ACGAGTTTTTTAAGGTCCCTTGGGAAAGGGGGGGGGTAACAAAGGGGATAA AGGACTTTCCCTTTTTGGGGGCGGGGGAAAACCGGGGAACCCCCTTAAAAG GGAAATTCCGGAAACCGGGGTAAACCGGAAAATCAGTTCCCGCCGGGAATT TAAAGGGGGCTTTTGTTCCCATTACAAAGGACCCCGGGGCCTTAGCTAGTTG GGAGGTAACGGTCACCAGGGGACGATGGTAGCGACCTGAGAGGGGATCGG CCCCCTGGGATGAGACACGGCCCAGATTCCTACGGGAGGCAGCAGTAGGAA TCTTCCGCAATG
TH.20	AAGTGAAAAACCCACGGCTAAACAGTGGAGGGCCATTGGAAACTGGAGGAC TTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGA CGCTGAGGTGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGT CCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGTTTCGCGCCCCTTAGT GCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCT GAAACTCAAAAGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTT TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACACC CCTAGAGATAGGGCATTCCCTTCGGGGAC
TH.15	GCTATACATGCAAGTCGAGCGGATCTTTTAAAAGCTTGCTTTTAAAAGATTAG CGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAA CTCCGGGAAACCGGGGCTAATACCGGATAATTCTTGTCCTCTCATGGGGACA AGCTGAAAGACGGCTTACGCTGTCACTTACAGATGGGCCCGGGGCCCAATT GAATTGGTTGGGGAAGTTATTGGCTTCCCCCAAGGCAAACGATTGGGTAGCC GACCCGGGGGGGGGTGATCCGCCCCCCCTGGAATTGGAACCCGGCCCCAGA CTCCTTCGGGAGGCAGCAGTAGGGAATTTTTCCGCCAATGGACGAAAAGTCT GACGGAGCAACGCCGCGTGAGCGATGAAAGGCCTTCGGGTCGTAAAGCTCT GTTGTTAGGGAAGAACAAGTATCGGAGTAA

Molecular analysis of phylogeny. Figure 1 presents the 16S rRNA gene sequences from active isolates of active TB.07, TB.12, TH.15, and TH.20. Homology analysis using BLAST searching can be seen in Figures 2, 3, 4 and 5.

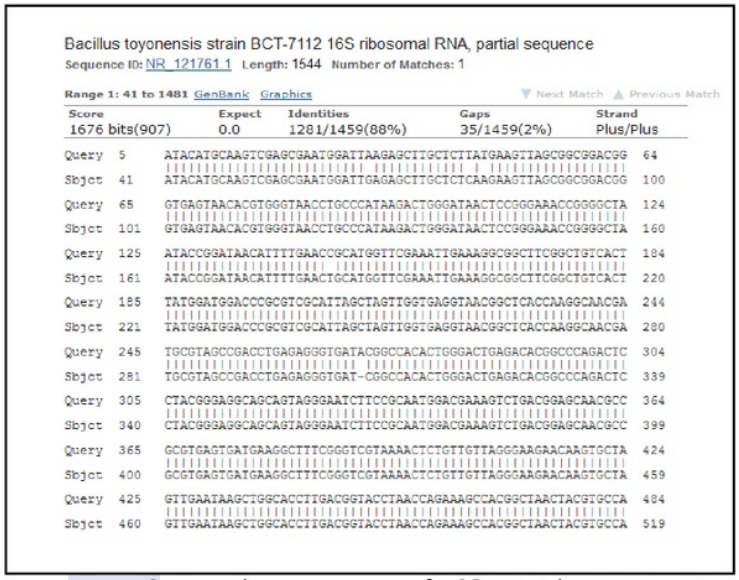

Figure 2. Homology sequence of 16S rDNA bacteria TB 7.

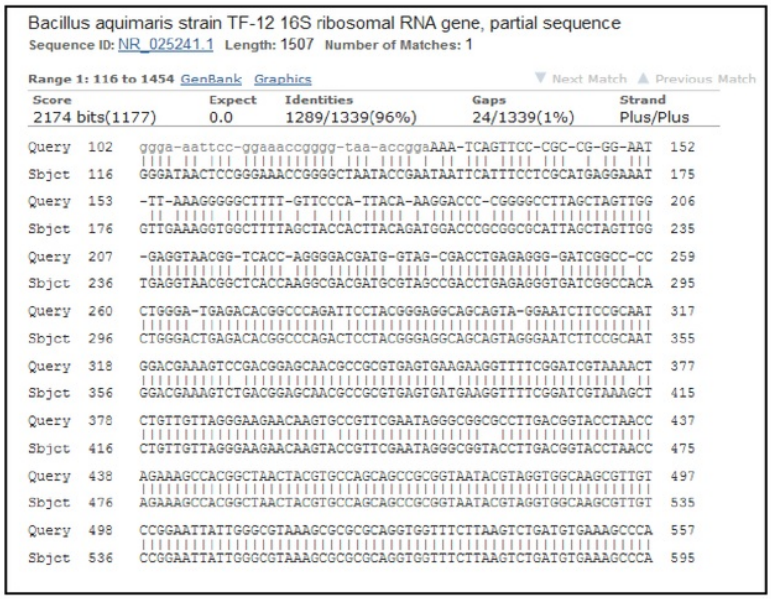

Figure 3. Homology sequence of 16S rDNA bacteria TB.12.

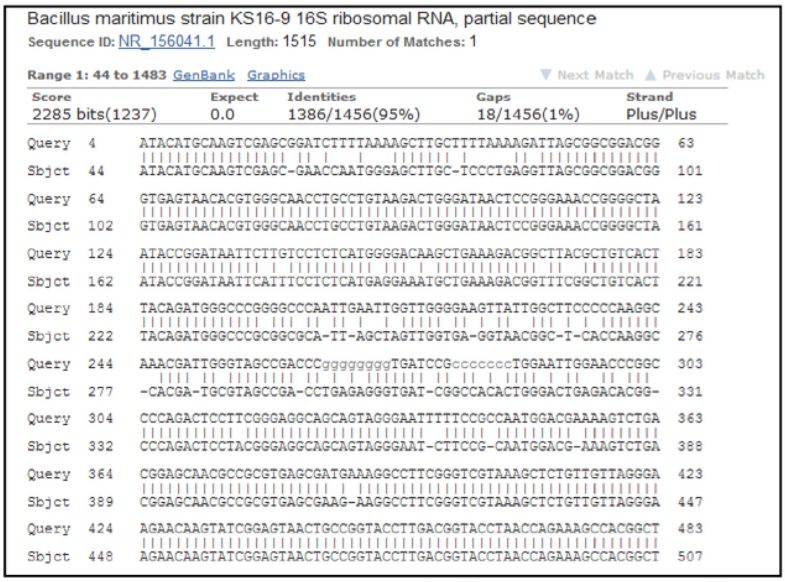

Figure 4. Homology sequence of 16S rDNA bacteria TH.15.

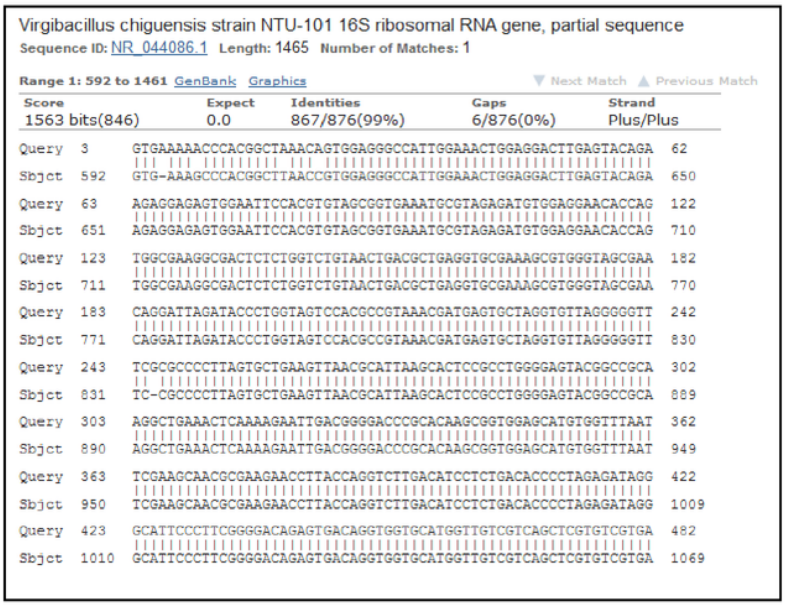

Figure 5. Homology sequence of 16S rDNA bacteria TH.20.

Homology of BLAST searching showed that isolate TB 7 has the highest similarity percentage with *Bacillus toyonensis* strain BCT-7112 (99%), isolate TB 12 has the highest similarity percentage with *Bacillus aquimaris* bacteria strain TF-12 (96%), TH15 isolates have the highest similarity percentage with *Bacillus maritimus* bacteria KS 16-9 (95%) and TH 20 isolates have the highest similarity percentage with the *Virgibacillus chiguensis* NTU-101 strain (99%) (Table 5).

Table 5

BLAST homology of symbiotic bacterial isolates

Isolate	*Relative match*	*Homology (%)*	*Access number*
TB 7	*Bacillus toyonensis* BCT-7112	99	NR_121761
TB 12	*Bacillus aquimaris* TF-12	96	NR_025241
TH 15	*Bacillus maritimus* KS 16-9	95	NR_156041
TH 20	*Virgibacillus chiguensis* NTU-101	99	NR_044086

Genotypic sequence analysis was performed using Clustal X. The neighbor-joining method used to obtain phylogeny tree construction can be seen in Figure 6. DNA sequences from sea cucumber symbiotic bacteria with close relatives of reference strains in outgroup organisms *Rothia mucilaginosa* X87758 as outgroup organism can be seen that TB isolates 7 have the closest kinship with *B. toyonensis* strain BCT-7112, TB isolate 12 has the closest kinship with *B. aquimaris* TF-12 strain, TH 15 isolate has the closest kinship with *B. maritimus* KS 16-9 strain, TH 20 isolate has the closest kinship with *V. chiguensis* strain NTU-101.

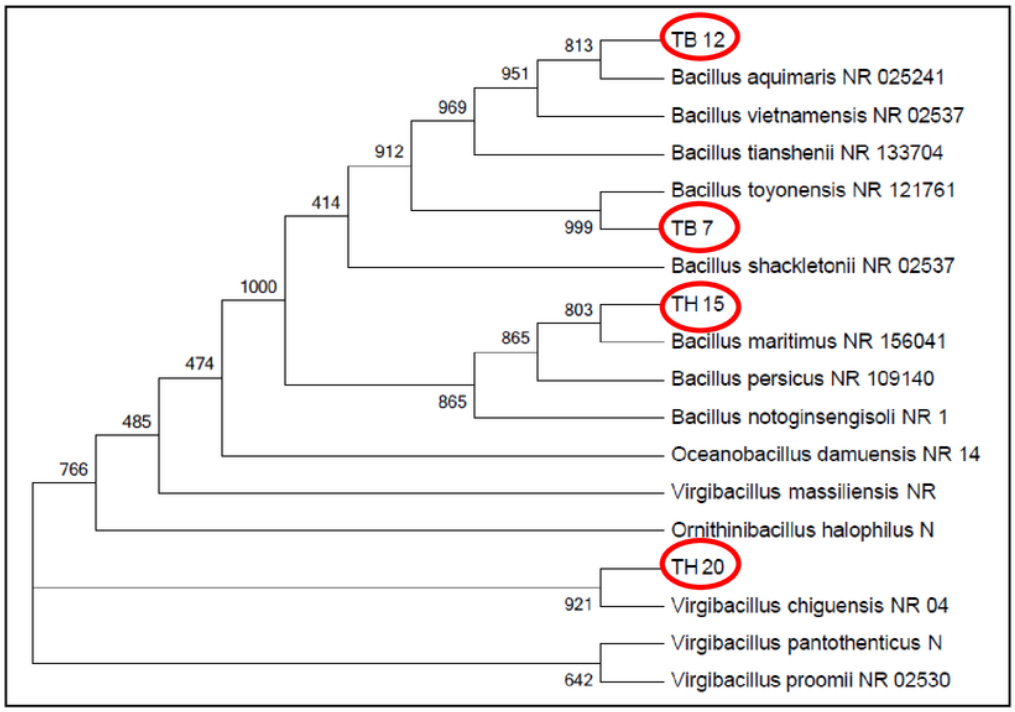

Figure 6. Phylogenetic trees of anti-MDR mollusk bacteria and reference strains obtained from the 16S rDNA database.

B. toyonensis bacteria BCT-7112 strain was found in the sea cucumber gut and symbiotic in its digestive organ. Sea cucumbers are benthos biota. The bacteria found in the sea cucumber floats, may be related to the nutrients consumed by sea cucumbers. As a filter feeder, sea cucumber takes food from it's around habitat. Sea cucumbers such as *H. atra* and *H. leucospilota* are types of benthos that live in sand environment, so it is suspected that sea cucumbers take food that is around the sand and including bacteria found in the stomach are bacteria that are also found in sand or sediment. Such as symbiotic bacteria *B. toyonensis* strain BCT-7112, *B. aquimaris* strain TF-12, *B. maritimus* strain KS 16-9 and *V. chiguensis* strain NTU-101 found in the stomach of sea cucumbers *Holothuria atra*

and *H. leucospilota* are types of bacteria found in sea sediments (Okaiyeto et al 2015; Waghmode et al 2017; Pal et al 2017; Wang et al 2018).

B. toyonensis is a bioflocculant-producing bacterium, isolated from marine sediment samples in the environment in Eastern Cape Province of South Africa showing flocculating activity above 60% for suspension of clay kaolin. The battery forms gram-positive spores which form an independent homogeneous branch in the genus *Bacillus*. This condition has tremendous economic interests, for example *B. toyonensis* BCT-7112 bacterial spores have been used in animal nutrition in some regions of the world (Okaiyeto et al 2015). The discovery of the symbiotic bacteria *B. toyonensis* BCT-7112 from the sea cucumber stomach is very promising information in the field of food and technology.

B. aquimaris is a candidate bacterium in osmo-adaptive biotechnology for some reasons. These bacteria are known to contain surfactant source, and its enzymes are capable to reduce the molecular weight of polysaccharide compounds and osmotic stress responses (Waghmode et al 2017).

The bacteria *B. maritimus* strain KS 16-9, which is found to be symbiotic in the sea cucumber stomach, feed on bacteria marine sediment by filter feeding. This type of bacteria is gram-strain-positive, rod-shaped, endosporic-forming (bulging sporangia) and aerobic. Pal et al (2017) reported that due *B. maritimus* wide tolerance to temperature and pH, have a negative reaction to hydrolysis of casein, starch, and gelatin, this *B. maritimus* bacteria are now becoming research of interest in the field of biotechnology.

V. chiguensis bacteria are gram positive white bacteria and having flagella for movement. Because the bacteria are found in compost media, it is considered that bacteria function as bacterial decomposers. Its characteristics are very useful in biotechnology (Arumugam et al 2014).

Conclusions. The study concluded that the consortium bacteria from four symbiotic bacteria, namely *B. toyonensis* strain BCT-7112, *B. aquimaris* strain TF-12, *B. maritimus* strain KS 16-9 and *V. chiguensis* strain NTU-101 have potential to produce antiseptic materials.

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