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Short Communication

Antibacterial potential of the symbiont red algae *Eucheuma cottonii* originated from Banten Bay Waters, Indonesia

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ABSTRACT:

Symbiotic bacteria are microorganisms that host plants with mutualistic symbiosis enabling them to produce compounds, especially antibacterial compounds. This research aimed to isolate and select symbiont producing antibacterial compound. Researchers conducted an examination to determine antibacterial activity using paper disc diffusion method. Symbiotic bacteria's antibacterial activity examination result exhibited zone of inhibition up to 2.55 mm against *Escherichia coli*. On the other hand, zone of inhibition against *Staphylococcus aureus* was 7.21 mm. Microscopic observations and biochemical examinations of the isolate EU-A revealed Gram-positive, rod-shaped and acid resistant bacteria that do not form endospore. Molecular analysis results on 16S rDNA revealed genus *Lactobacillus* in species *Lactobacillus plantarum*.

Keywords:
Euchuema cottonii, Symbiont bacteria, Antibacterial activity.

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INTRODUCTION

Eucheuma cottonii seaweed is a member of the class Rhodophyceae. It is generally grown on a substrate of sand and coral fragments (Anggadiredja *et al.,* 2008). Seaweed has primary and secondary metabolite contents. The primary metabolite content such as vitamins, minerals, fiber, and carrageenan, an alginate used as cosmetic ingredients for skincare. In addition, primary metabolites possesses economic value and seaweed produce secondary metabolites with various bioactivities such as antibacterial, antiviral and antifungal (Zainuddin and Malina, 2009).

Seaweeds naturally associated with a wide range of bacteria. Symbiotic plants and bacteria form colonies on rhizospere (rhizobacteria), phyllosphere (epiphyte), and in the tissue (endophyte). Endophyte exists within the tissue, therefore, it is protected from the extreme environment and microbial competition from the host plants (Mano, 2007). Bacterial endophyte aid in plant growth such as producing phytohormone, increase resistance to the pathogen and parasite, aiding nitrogen fixation and production of antibiotics (Feng *et al*., 2006; Lisdayanti, 2013).

Utilization of symbiotic microorganism has drawn attention because of their ability to produce active compounds. These active compounds used to defend themselves from pest attacks and environmental hazards. There had not been much research data which explored the antibacterial compounds from red algae symbiotic bacteria as well as other types of algae as raw material for medicinal purposes.

This research aims to identify the bacterial stock of the red algae *Eucheuma cottonii* symbiotes located in Fisheries University Laboratory and determine the antibacterial activity potential of the *Eucheuma cottonii* symbiotes of bacteria selected by paper disc diffusion method.

MATERIALS AND METHODS

Materials

The materials used in this research is red algae *Eucheuma cottonii*, a pure culture of bacteria *Staphylococcus aureus* and *Escherichia coli*, nutrient broth (Oxoid), Plate Count Agar (PCA) (Oxoid), Mueller Hinton Agar (MHA) (Oxoid), and sterile sea water.

Identification of isolates producing antimicrobial compounds

EU-A isolates were identified by observing colony morphology, gram staining, spore staining and acid-fast stainning as well as biochemical examinations such as carbohydrate fermentation examination (glucose, arabinose, mannitol), urease, catalase, motility, citrate reduction and gelatin. Bacterial identification was conducted based on the Cowan and Steel's manual for identification of medical bacteria, third edition, 2003 (Cowan *et al.,* 1993).

Examination for antimicrobial activity

The supernatant testing of symbiont bacteria for inhibitory growth of *E. coli* and *S. aureus* was performed by the agar diffusion method (Hudzicki, 2009). Symbiont bacterial culture that has been identified are grown in nutrient broth and incubated for 3-4 days. Centrifugation process was conducted at 3,000 rpm for 1 hour to separate supernatant and residue. The MIC (Minimum Inhibitory Concentration) for supernatant as free cell extraction isolate symbiont bacteria *Euchema cotonii* in agar dilution is 40 µg/ml. After preparing supernatant, inoculated sterile 20µl supernatant were placed on the paper discs. *E. coli* and *S. aureus* are grown on the Mueller Hinton media at different petri dish using pour plate technique. 1 ml of bacteria suspension was taken and inoculated in 10 ml MHA and then poured into a petri dish and rocked slowly to mix with bacteria cultures along with gelatin. After the compound solidified, paper disc containing inoculat-

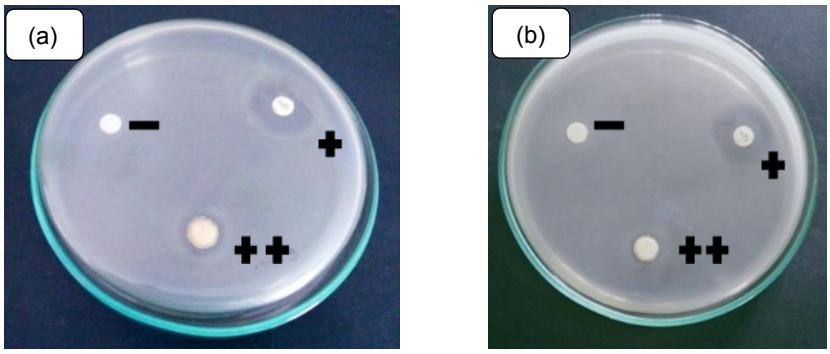


Figure 1. Antibacterial activity examination result on supernatant against *Staphylococcus aureus* (a) and *Escherichia coli* (b)

ed supernatant were placed on the media surface, with the positive control of chloramphenicol 10 µg/ml negative control was maintained as a petri dish with MHA without inoculation of the supernatant. Incubation was conducted at 37^{o}C for 24 h, 48 h and 72 h. Inhibitory potency is determined by measuring diffusion diameter using the caliper with the accuracy of 0.05 mm. The microbial analysis is maintained for three replicas.

Identification of phenotype and genotype of symbiont bacteria

Identification of phenotype and genotype of symbiont bacteria conducted by general bacterial identification (Lay (1994) and identification keys from Cowan *et al*. (1993).) which followed observing cell morphology by gram staining, spore staining, and Ziehl-Neelsen staining and various biochemical tests (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase). The initial selection of isolates from mixed cultures was carried out after enrichment and planting samples on the agar medium in pour plating. After 24 h and 48 h of incubation at 37°C, the growth was observed.The data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from *Euchema cottonii*.

Samples of bacteria grown on PCA medium were incubated at 37°C. for 3 days and then checked for purity and then used for the molecular identification process. Identification was performed using molecular analysis based on 16S rDNA fragments in bacteria. Isolation of bacterial genomic DNA was performed using PCR colony method (Packeiser *et al.,* 2013). Cells from a single colony on the surface of a solid medium were taken with a loop and suspended into 50 µL of nuclease free water. Cell lysis was performed with a divortex suspension for 10 seconds and incubated at 98°C for 5 minutes. Lysate is further spin down to separate supernatant and cell debris. The supernatant was taken and used as a DNA template on PCR amplification.

Symbiont bacteria species was determined by molecular testing. The DNA of the symbiont bacteria isolates was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR

Table 1. Antibacterial activity of supernatant EU-A examination result

S. No	Supernatant/ Comparison	Inhibitory zone diameter (mm)						
		S. aureus			*E. coli*			Result
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	
1.	Supernatant **(++)**	7.21	7.21	7.19	2.55	2.47	2.38	+
2.	Positive control (Chloramphenicol) **(+)**	16.06	16.04	16	13.63	13.60	13.57	+
3.	Negative control (Nutrient broth) **(-)**	0	0	0	0	0	0	-

product of about 1400 base pairs. Molecular identification was done through partial genetic analysis of 16S rDNA. DNA extraction was performed using the modified GES method (Pitcher *et al*., 1989). PCR Amplification on 16S rDNA using **primer 9 F:** 5`- AAG GAG GTG ATC CAG CC - 3` and **primer 1541 R:** 5` - GAG TTT GAT CCT GGC TCA G - 3` (White *et al*., 1990; O`Donnell, 1993). The analysis of nitrogen base sequence readings was done using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The next sequenced raw data was trimmed and assembled using the BioEdit program (BIOEDIT, 2005). Sequencing data that has been assembled in BLAST with genome data that has been registered in DNA Data Bank of Japan (DDBJ, 2017).

RESULTS AND DISCUSSION

Antibacterial activity of disc diffusion method

Based on the data presented in Table 1, the supernatant obtained in general are able to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*. The area of the symptomatic supernatant inhibition zones of *S. aureus* were 7.21 mm, 7.21 mm and 7.19 mm, while inhibition zones of *E. coli* were 2.,55 mm, 2.47 mm and 2.38 mm. According to Kusumadewi (2004), a measured inhibition zone of less than 10 mm shows weak activity and strong activity if the inhibition zone is greater than 15 mm. Testing of antibacterial activity of the symbiont bacteria supernatant obtained was still far from the results of the antibiotic activity of the chloramphenicol. This is because the antibacterial compound of the extracted symbiont bacteria was a supernatant containing secondary metabolites. However, the test results provide clear evidence of antibacterial activity. This is demonstrated by the existence of a clear zone around a paper disc that proves that supernatant bacteria of the symbiotes algae contain antibacterial compounds that can inhibit bacteria to some extent. Inhibition zone formed by the supernatant on *Staphylococcus aureus* and *Escherichia coli* bacteria is presented Figure 1.

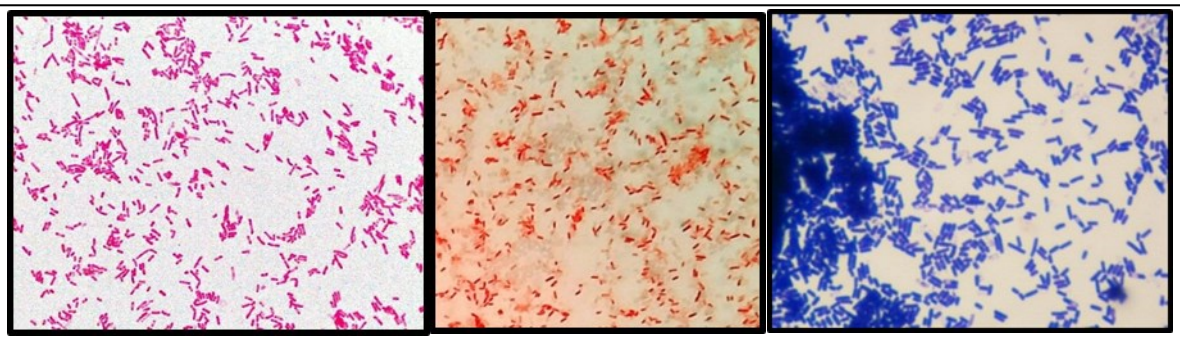
Table 2. Symbiotes EU-A bacterial profile

S. No	Examination	Results
1	Shape	Rod
2	Endospore	Does not form endospore
3	Acid-fast	Acid resistant
4	Glucose	Positive
5	Mannitol	Positive
6	Arabinose	Negative
7	Urease	Negative
8	Citrate	Negative
9	Gelatin	Positive
10	Motility	Negative
11	Catalase	Negative

Based on the obtained result, antibacterial activity is better against gram-positive bacteria. This is due to the cell walls structure is Gram-positive bacteria. 90% of the cell wall is composed of peptidoglycan layer, while the other is a thin layer of teichoic acid. Teichoic acid units containing glycerol or ribitol bound to each other by phosphoric ester and typically contain other carbohydrates as well as D-alanine (Fardiaz, 1992). In addition to containing teichoic acid, cell walls of Gram-positive bacteria also contain teichuronic acids which are negatively charged. These molecules are microfibril that makes it easier to form lipoteichoic adhesions (Madigan *et al*., 2003.).

After an incubation period of 3 x 24 hours, there was a decrease in the diameter of the zone of resistance against the bacteria *Staphylococcus aureus*. This indicates that bioactive compound contained in the supernatant is bacteriostatic and able to turn it off. According to Wattimena (1991), a bacteriostatic antimicrobial is potential to inhibit the growth of bacteria examination and do not switch it off until 48 hours within regional barriers back overgrown by bacteria, thus decreasing the inhibition diameter.

The ability of supernatant in inhibiting bacteria indicates the barriers that zone diameter is much smaller



Shape : Rod gram negative **No endospore** **Acid resistant**

Figure 2. Gram, endospore and acid fact staining results of the bacteria identified

than the positive control, this may happen because the diffusion of active ingredients on the media that progress was slow and had low concentrations of the active substance content, therefore fraction does not inhibit optimally (Cappucino and Sherman, 1992).

Identification of bacteria producing symbiotic antibacterial compounds

Bacterial identification was conducted by observing the morphology of the colony, cells and biochemical examinations include carbohydrate, urease, citrate, gelatin, motility and catalase (Table 2).

Morphology of isolate

The morphology of symbiotic bacteria are microscopic in the form of Gram positive rods, acid-resistant and do not form spores (Figure 2). The bacteria form colonies, round-shaped and milky white color.

Fermentation of carbohydrates

Fermented carbohydrates (glucose and mannitol) exhibits the color change from red into yellow (Figure 3). The color change is not accompanied by a gas that is formed on Durham tube. According to Lay (1994), in the process of fermentation, bacteria were grown in liquid media containing carbohydrates resulting in a form of acid. The resulting acid lowered the pH of the culture medium. The formation of lactic acid was marked by a change of the media turning yellow in color. The color change followed by formation of gas in the Durham tube is a mixed acid fermentation and fermented in the absence of color change but gas formed at Durham tube indicates the presence of alcoholic fermentation. Thus, the examination results of carbohydrate fermentation (glucose and mannitol) indicates EU-A bacteria can ferment carbohydrate through lactic acid.

Gelatin hydrolysis

Gelatin hydrolysis helped to detect the gelatinase enzyme. Gelatinase examination results gave a positive result which means there are gelatinase

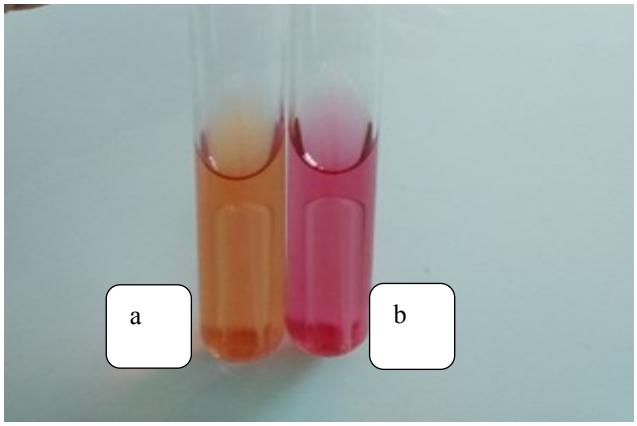


Figure 3. Result of carbohydrate fermentation (a: sample/ b:blank)

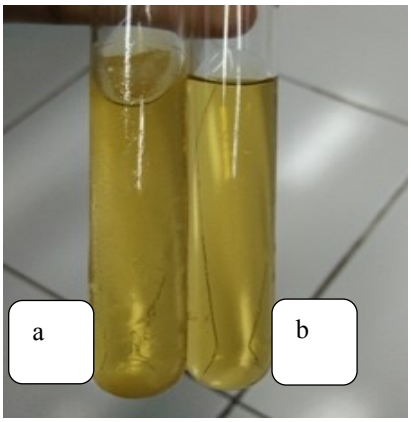


Figure 4. Result of gelatin hydrolysis (a: sample/ b:blank)

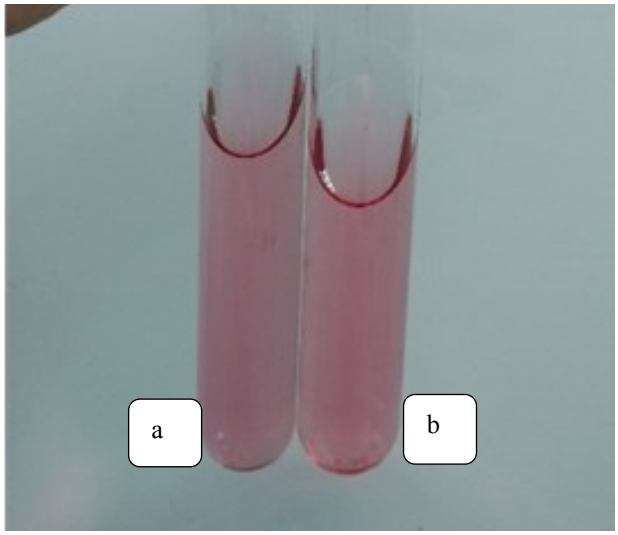


Figure 5. Result of negative urease test (a: sample/ b:blank)

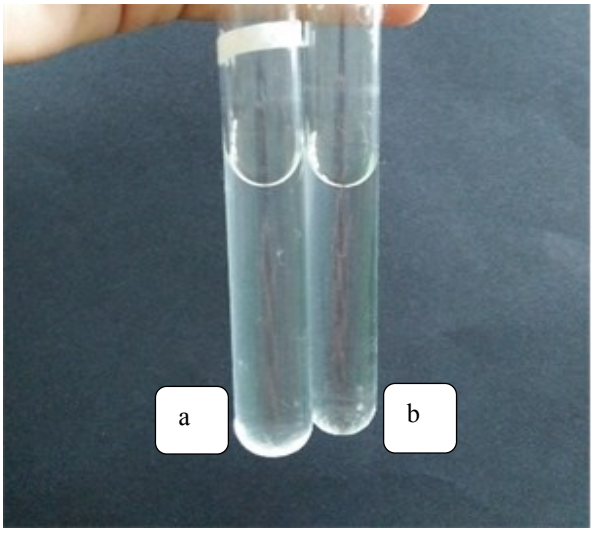


Figure 6. Result of negative citrate analysis (a: sample/ b:blank)

enzymes in the bacteria symbiotes in EU code-A (Figure 4). Therefore it could be inferred that EU-A isolates are proteolytic, one of its ability is being able to decipher gelatin into amino acids.

Urea hydrolysis

Several microorganisms are capable of producing the enzyme urease which catalysis the reaction of urea into ammonia and CO_2. Urease enzyme activity can be observed by growing microorganisms in culture medium containing urea and pH indicators. When urea hydrolyzed, $NH4^+$ accumulated in the culture media and cause pH to become alkaline. Urease examination gave a negative result which is indicated by the absence of color change from purple to red (Figure 5).

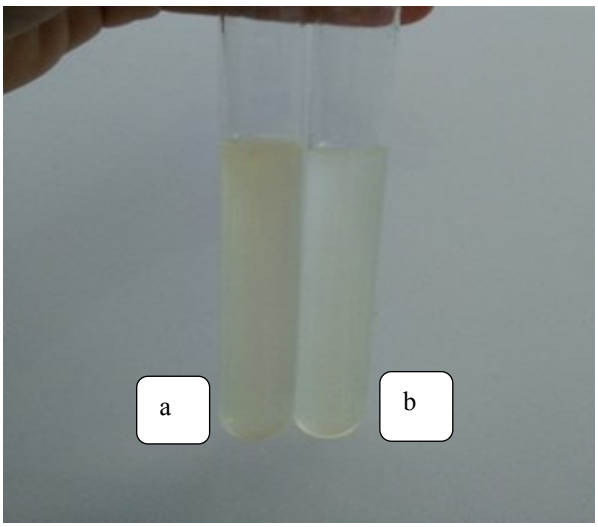


Figure 7. Resault of motility test showing non-motile bacteria (a: sample/ b:blank)

Citrate examination

Citrate is used to examine the ability of microorganisms to use citrate as a sole source of carbon and energy. This examination was done using media kosher citric acid which contains no indicators. Citrate examination results showed negative results and are characterized by the absence of turbidity on medium (Figure 6). This exhibits the symbiotes EU-A bacteria is not capable of using citrate as the sole source of carbon.

Motility test

Motility tests revealed non motile bacteria. According to Lay (1994), a negative reaction on motility test exhibited reveals that bacterial isolates do not have motile growth (Figure 7).

Catalase test

A negative catalase reaction was observed. According to Ernawati (2008), a negative reaction on catalase examination exhibits that bacteria isolates do not have to possess enzyme catalase which can degrade the hydrogen peroxide (H_2O_2) into water and O_2. Cowan

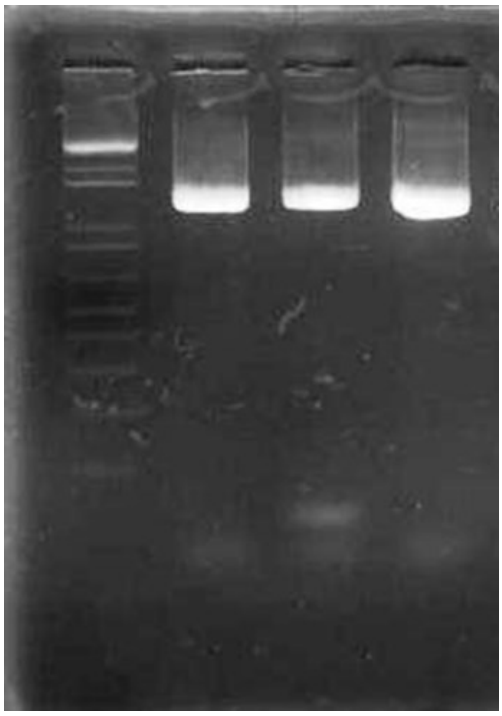


Figure 8. The PCR 16S rDNA visualization results of isolate EU_A

Caption:

- The sample PCR product (3) EU_A is 1500bp, (-) negative control
- Ladder DNA 1kb plus

100, 200, 300, 400, 500, 650, 850, 1000, 1650, 2000, 3000, 4000 bp

and Steel's identification key (1993) indicated that there are five types of bacteria that are thought to have a similar character i.e. *Brochothrix, Erysiplohrix, Lacto-bacillus, Arcano*bacterium and *Arachina*.

16S rDNA molecular examination

The EU-A isolate showing the largest inhibition zone was then tested molecularly. 16S rDNA molecular examination was conducted to determine the symbiotic bacterial species. Molecular identification was done through partial genetic analysis of 16S rDNA. The PCR amplification results of the 16S region of ribosomal DNA of the bacteria were given in Figure 8. Sampled PCR products were visualized using a gel documentation system measuring 1500 bp and negative controls did not contain the following DNA bands. The DNA bands used were relevant to the resulting PCR product of about 1500 base pairs. The sequence of nitrogen bases sequenced from the EU-A bacteria isolate can be seen in Figure 9.

Based on secondary data obtained from InaCC LIPI (Indonesian Culture Collection), the sequence analysis of 16S rDNA isolates of EU-A were

TGGTTCCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCCATGGTGTGAC-
GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATT
CCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTAGCTTAC
TCTCGCGAGTTCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCA
TGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACCAGAGTGCCCAACTT
AATGCTGGCAACTGATAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA
GCTGACGACAACCATGCACCACCTGTATCCATGTCCCCGAAGGGAACGTCTAATCTCTTAGATTTGC
ATAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGCTCCACCGCTTG
TGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAATGCTTAAT
GCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCATTCATCGTTTACGGTATGGA
CTACCAGGGTATCTAATCCTGTTTGCTACCCATACTTTCGAGCCTCAGCGTCAGTTACAGACCAGAC
AGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATTTCACCGCTACACATGGAGTTCCACTG
TCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGCACTTCTTCGGTTGAGCCGAAGGCTTTCACATC
AGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTA
TTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATACCGTCAATACCTGAACAGT
TACTCTCAGATATGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGC
GTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCC
GTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGCTACGTATCATTGCCATGGTGAGCCG
TTACCCCACCATCTAGC TAATACGCCGCGGGACCATCCAAAAGTGATAGCCGAAGCCATCTTTCAA-
GCTCGGACCATGCGGTCCAAGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCCGCTTC
TGGGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAATCATGATGCAAGCA
CCAATCAATACCAGAGTTCGTT

Figure 9. The sequence of nitrogen bases sequenced from the EU-A bacteria isolate
A= Adenine, T= Thiamine, G=Guanine, C=Cytosine

identified as *Lactobacillus plantarum* which has similarity in 99%, at a max score of 2619 (total score 2619), query 100% coverage, the E value 0.0 against bacterial taxa.

CONCLUSION

Antibacterial activity study results from bacteria of the symbiotes EU-A exhibits the zone of inhibition of 2.55 mm against the bacteria *Escherichia coli* while cruising inhibition against the bacteria *Staphylococcus aureus* at 7.21 mm indicating bacteriostatic effect. Based on the morphological and biochemical characterization of the isolates into A include EU-Group of Gram-positive rod-shaped, not acid and inability to form endospore, 16S rDNA examination results indicate that isolate EU-A is a member of genus *Lactobacillus* and species *Lactobacillus plantarum.*

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