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Antibacterials Potential Symbiont Bacteria OOf Brown Algae
(<i>Turbinaria conoides</i>) Obtained fFrom Banten Bay
Serang District - Province Of Banten Indonesian Waters

Niken Dharmayanti, <u>Aef Permadi, Yuliati H Sipahutar, R</u>esmi Rumenta Siregar, Arpan Nasri Siregar, Randi Bokhi Salampessy<mark>, Sujuliyani,</mark> Arma<u>-Anti</u>anti

Sekolah Tinggi Perikanan, Jakarta, Indonesia-Study program<u>of Fish Processing Technology, Jakarta Fisheries</u> Technical <u>University, Pasar Minggu</u> 12520, South Jakarta, Indonesia

ABSTRACT

Brown seaweed has the potential to produce bioactive compounds. It has been shown that the bacteria associated with seaweed are involved in the production of metabolites associated with their host. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes (Kalaivani et al., 2016). In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were brought from Lima Island, Serang District, Banten Bay. Symbiont bacteria withtested for antibacteria all activity, were isolated using the bioassay test method. A total of 14 isolates were isolated, 6 of which came from the outsideexternal tissue, while 8 isolates came from the inside of the algaeintemal tissue. Through the antagonistic test, 7 isolates showed inhibitory activity against <u>StaphylococcuS-aureus</u> and 1 isolate bacteria-showed the best-inhibition against both *S.aureus* and *E.coli*. Selected isolates have the ability to inhibit *S.aureus* after diffusion paper disc tested. Phenotypic and genotypic identification showed that the species symbiont bacteria of *Turbinaria conoides* isomators.

19 Keywords: bioassay, antagonistic, diffusion paper disc, Lactobacillus plantarum.

1. INTRODUCTION

Seaweed is an algae that lives in the sea and belongs to the division of *thallophyta*. The classification of seaweed based on pigment content consists of 4 classes, namely green seaweed (*Chiorophyta*), red seaweed (*Rhodophyta*), brown seaweed (*Phacophyta*) and blond seaweed (*Chirysophyta*) (Suparmi and Sahri, 2009). Indonesia is the largest producer of seaweed (*Phacophyta*) and blond seaweed (*Chirysophyta*) (Suparmi and Sahri, 2009). Indonesia is the largest producer of seaweed in the world (FAO 2016) cultured in nearshore coastal regions. In addition to its primary economical content, the secondary metabolite content of seaweed has the Seaweeds potential of being a producerpoduce of diverse bioactive metabolites with vast activity as antibacterial, antiviral, antifungal and cytotoxic properties (Zainuddin and Malina, 2009 in Siregar et al., 2012). Bacteria usually live on a host by performing a mutually beneficial symbiosis (Sahara et al., 2013). It has been shown that the bacteria associated with seaweed as epiphytes or endophytes are involved in the production of metabolites that together with their host. Microbes can be present as a living symbiotic in union with various marine algae as epiphytes or endophytes. (Sartika et al., 2014, Kalaivani et al., 2016). Symbiont bacteria isolates in algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of the form of symbiotic mutualism. Algae provide the places needed sites and nutrients the bacteria need, while the bacteria encourage growth and protect the algal surface against pathogens (Hollants et al., 2012 in Sartika et al., 2014). Seaweeds can secrete secondary metabolites with antibacterial properties (Burgesset et al., 1999; Armstrong et al., 2001; Yanet et al., 2003 in Nofiani, 2005).

Brown algae and other types have been extensively analyzed for their antibacterial and antifungal activity (Bhakuni and Rawat, 2005). Previous research on brown algae *Sargassum sp.* Has been shown to have antimicrobial potential of bioactive proteins from bacteria symbiotic with it (Sartika et al, 2014) and the potential of bacterial algaeHere we evaluate the properties of the brown algae *Turbinaria conoides* in producing bioactive compounds in inhibitingincluding the inhibition of pathogenic bacteria Urinary Tract Infection (UTT)human pathogens (Kalaivani et al., 2016). *T. conoides* is a tropical marine alga widely distributed in coastal waters in SE Asia. We chose this alga following extensive trials on other common macroalgae including *Sargassum* spp. and *Euchema cottoni*.

The study focused on identification of macroalgae found in the sampling sites, isolation symbionts
 of the *Turbinaria conoides*, selection of symbiotic bacteria isolates, testing of antibacterial potency by
 diffusion of paper dises, and identification of the phenotype and genotype *Turbinaria conoides* symbiont bacteria.

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31 Materials

MATERIALS AND METHODS

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32 The materials used in this research are Turbinaria conoides,., pure cultures of S.aureus, pure culture of E.coli, 33 aquadesh, nutrient broth (Oxoid), plate count agar (Oxoid), mueller hinton agar (Oxoid), sterile sea water, 70% alcohol, 95% 34 alcohol, spirtus, crystal violet, iodine, safranin, immersion oil, carbolfuesin dyes, alcoholic acid, methylene blue, malachite green solution, safranin solution, filter paper, tissue, cotton, brown paper, paper disc, matches.

35 36 The equipments used are petri dishes, test tube, beaker, measuring cup, preparatory glass, measuring pipette 37 38 (omnipipette), dropper pipette, tip pipette, micro pipette, mortar, tube rack, scales (vibra), inoculation loops, Spatula, bent glass, sterile plastic, magnetic stirrer, bunsen, hotplate (thermo scientific), scooter, microscope (olympus), scissors, tweezers, 39 autoclave (kemoto scientific), incubator (memmert), oven (memmert), shaker (thermo Scientific), refrigerator (selecta), 40 laminary air flow (telstar), ohp markers, elastic bands, centrifuge (eppendorf), eppendorf tube, vortex mixer 41 (heidolph).Application GPS mobile phone

43 MethodsProcedures

44 Sampling

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45 Samples of Turbinaria sp. (about 1 kg wet weight) was-were taken from Lima island (S: --6.0010516; E: 46 106.153804) around 1 kg for determination in the morning around 7 at low tide allows the position of algae 1 meter below 47 the water on the sidelines of the reef directly dried in the bundle of the island. Sampling was continued at 16 o'clock when 48 the low tide and taked 500 grams for antibacterial test and kept in the plastic pouch and immediately filled with seawater, with seaweed : water ratio of 1: 2 until submerged, when it already arrived on Serang filled with oxygen 1 : 2 more air. The 49 50 seweed stayed in the plastic with oxygen from Serang until Jakarta for a night and started done in the laboratorium in the 51 morning.Samples were maintained in fresh seawater for laboratory analyses within 24 hour, of collection.

52 53 **Identification and Determination of Macroalga**

Dried Turbinaria sp. found in predetermined location or stations were recorded and identified by macroalgae type 54 55 56 57 58 59 through algae base associated with observed macroalgae characteristics. The type of macroalgae used in this study was a genus of Turbinaria sp. The location is in the waters of the island of Lima, Banten Bay, Serang regency. The macroalgae determination used has done in LIPI Oceanography. Identification was performed on the method of form specification with reference to algae identification guidelines (Brigham et al., 2004; Lee, 2008)

Isolation of Symbiont Bacteria Producing Antibacterial Compounds

Surface of Algae: Epibionts were extracted from 15 grams of algae by rinsed rinsin with 30 mL of sterile sea water. 60 61 The rinse water is was put into incubated in 30 mL of nutrient broth medium-then shaken by shaker_at room temperature for 24 hours. Inside of algae: as many as Bioactive compound _15 grams of algae were rinsed with 30 mL of sterile sea water, were 62 63 64 65 extracted by erushed crushing 15 g of algafinely using mortal-with a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension is then fedwas incubated into-with 30 mL broth nutrient medium and shaken by shaker aat room temperature for 24 hours.

After extraction process, Fihe refreshed samples of in the 30 ml broth nutrient medium were diluted into 9 ml broth 66 nutrient sterile by 10⁻¹ up to 10⁻⁵. Each dilutions was were grown on a plate count agar medium by incubate them at 37 °C 67 for 2 x 24 hours. After incubating the petri dishes which were contain samples from each dilution, then the colonies bacteria 68 from alga would appear. The colonies bacteria producing antimicrobial compounds are were characterized by a clear zone 69 70 around the colonies. Furthermore, the colonies with stable inhibition zones were collected -by and isolating themted on slant agar medium, with a clear code.

71 72

Selection of Symbiont Bacteria Isolates Antagonistically against Pathogenic Bacteria

73 74 To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria, a qualitative test was 75 76 77 78 conducted directly by scratching or bottling round the isolates on the surface of the media that has been dispersed with test bacteria (Eschericia -coli and Staphylococcus-aureus). Then-Media were incubated for 2 x 24-48 hours at 37 °C. Each scratching round of isolates was then marked by its a unique code.

Inhibition zones were read as the point determined as those showing clear zones around the colony of simbiont. 79 bacteria isolates, the more clear zone of isolates in inhibit for both Escheriacia coli and Staphylococcus aureus, are the better 80 their activity. Strains -that showed maximum antagonistic effect againsts tested pathogens were choosed and marked by its 81 eodeidentified. Isolates that These ehoosen isloate with appropriate code which was formed a clear zone or has with the a 82 highest activity are waswere isolated and selected for - further antibacterial testing by paper disc and identification of 83 phenotype and genotype testin.g.

84 Antibacterial Potential Testing of Symbiont Bacterial Isolate by Paper Disc Diffussion

85 Testing inhibitory the supernatant of symbiont bacteria on the for inhibitory growth of E.coli and S.aureus was 86 performed by the agar diffusion method (Hudzicki, 2009) REFERENCE), Supernatant was obtained by separating the 87 filtrate and supernatant by centrifugation processcentrifuge for 1 hour, temperature at (25 °C and 3000 rpm). Paper discs 88 containing supernatant 40 µL and the negative control nutrient broth 40 µL which has allowed were dried left for 1 hour to 89 reduce the water excess-(dried), negative control nutrient broth 40 µL also has allowed for 1 hour to reduce the water excess

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90 (dried) and positive control chloramphenicol 0.01 mg/mL, were placed on the surface of the Mueller Hinton Agar Almedium 91 containing 1 mL test bacteria. Furthermore and incubated for 2 x 2448 hours at 37 °C. The supernatant diffuses from the 92 disc into the agar-in decreasing amounts the further it is away from the dise. If the organism is killed or inhibited by both 93 the supernatant and chloramphenicol as antibiotic positive control, there will be no growth in the immediate area around the 94 disc, this is called the zone of inhibition. The zone sizes awere compared up on a standardized to give a result of to assess 95 bioactivity as sensitive, resistant, or intermediate, te then It was observed and measured its in each case the resistance zone 96 where shows no colonies growth with by a ruler was measured by using ruler to the nearest mm.

98 Identification of Phenotype and Genotype of Symbiont Bacteria

99 In general, General bacterial identification was performed in accordance with the microbial analysis procedure in 100 the laboratory (Lay, 1994 and identification keys from Cowan and Steel (1993)) by performing followed colony characteristic observations on liquid medium and solid medium, observing cell morphology (gram staining, spore staining, 101 102 and Ziehl-Neelsen staining), and test Biochemistry test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and 103 catalase). The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria conoides samples on the agar medium in pour plating. Observation of medium incubated with temperature 37°C was done 104 105 at incubation time reached 24 hours and 48 hours. The data obtained from the bacterial isolate characterization were used to 106 estimate the type of symbiotic bacteria isolated from the *Turbinaria conoides*-seaweed. Determination of the type of bacteria 107 was performed based on identification keys from Cowan and Steel (1993). Symbiont bacteria species was determined by 108 molecular testing.

110 The DNA of the symbiont bacteria isolates was amplified using primers 9F and 1541R. The DNA bands used were 111 112 relevant to the resulting PCR product of about 1400 base pairs, The PCR reaction used a PCR machine (Eppendorf German) with a first predenaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C for 30 113 114 seconds, primary attachment at 50 ° C for 30 seconds and extension at 72 ° C for 90 seconds. After 30 cycles completed, followed by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular identification was done 115 116 through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989. Modified). PCR Amplification on 16S rDNA using Primer 9 F: 5' -- AAG GAG GTG ATC CAG CC-3' and Primer 1541 117 R: 5' - GAG TTT GAT CCT GGC TCA G - 3' (White et al., 1990; O'Donnell, 1993). The analysis of nitrogen base sequence 118 readings using-was performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied 119 120 The next sequenced raw data waswere trimmed and assembled using the BioEdit program Biosystems). (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data that has been were assembled in BLAST with genomic 121 data that has been registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/)

RESULTS RESULTS AND DISCUSSION

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125 The Result of Identification and Determination of Macroalga

The macroalgae observation area and the sample site obtained are determined based on the location coordinate point. The location of macroalgae observation S: 6.0010510; E: 106.1538040 has morfology characteristic as Cylindrical rods, erect, rough, there are traces of branching. Holdfast is a small disc with radial expansion root. The branches rotate around the main trunk. Leaves are unity consisting of stalks and sheets. It named *Turbinaria conoides*.



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Turbinaria conoides

Table 21.- and identification of isolates isolated into slant agar can be seen in Table 23.

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The Result of Symbiont Bacteria Isolation

The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria conoides samples on the agar medium in pour platting. Observation of medium incubated with temperature 37°C was done at incubation time reached 24 hours and 48 hours. When incubated, the individual microbial cells multiply so rapidly that within 18 to 24 hours a visible mass of cells is formed and is called a colony (Pelzcar and Chan, 1986).



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Tabel_21 Macroscopic forms of bacterial colonies

No	Colony code			Morp	bhology of colonies
NO	Colony code	Shape	Color	Edges	Elevation
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	TUL ² -A4-2	Round	White	Flat	Convex shiny
	TUL ² -B1-2	Round	White	Crooked	Convex shiny
	TUL ² -B2-2	Round	White	Crooked	Convex shiny
	TUD ⁴ -C1-2	Round	White	Flat	Convex shiny
	TUD ⁴ -C2-2	Round	White	Flat	Convex shiny
	TUD ² -D1-2	Round	White	Crooked	Convex shiny
0	TUD ² -D2-2	Round	White	Crooked	Convex shiny
1	TUD ² -D3-2	Round	White	Crooked	Convex shiny
2	TUD ² -D4-2	Round	White	Crooked	Convex shiny
3	TUD ⁵ -E-2	Round	White	Flat	Convex shiny
4	TUD ³ -F-2	Round	White	Flat	Convex shiny
forma					
he co	de of isolates TUL	TUD states th	e isolates origin	ating from the oute	r/inner algae

The grown From 40 s_Samples consisted of the inside and outside of the algae, as many as 20 petri for 2 replications

resulted in colonies with the inhibit zone of 14 colonies, 6 of which were from the outside epibionts, while the other 8 came from the inside of the algaealgal tissue. The results of identification of colonies grown on mixed cultures can be seen in

** The code of isolates $(^{2}), (^{2}), (^{3})$ states isolates obtained from the dilution *** The code of isolates $(^{2}), (^{3}), (^{3}), (^{3})$ states isolates obtained from the dilution *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed on the plate

163 **** The code of number 2 identifies the isolate obtained from the second repeat

164 165

Table_32. Identification of the isolates on slant agar

Solid medium Formatted Table Code of isolates No Shape Color Formatted: Indent: Hanging: 1,46 cm

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1. TUL ² -A1-2	C	MCH 124
	Spread	Milky white
2. TUL ² -A2-2	Spread	Milky white
3. TUL ² -A3-2	Spread	Milky white 🔸
4. TUL ² -A4-2	Spread	Milky white
5. TUL ² -B1-2	Rhizoidal	Cloudy white
6. TUL ² -B2-2	Rhizoidal	Cloudy white
7. TUD ⁴ -C1-2	Spread	Milky white
8. TUD ⁴ -C2-2	Spread	Milky white
9. TUD ² -D1-2	Rhizoidal	Cloudy white
10. TUD ² -D2-2	Rhizoidal	Cloudy white
11. TUD ² -D3-2	Rhizoidal	Cloudy white
12. TUD ² -D4-2	Rhizoidal	Cloudy white
13. TUD ⁵ -E-2	Spread	Milky white
14. TUD ³ -F-2	Spread	Milky white
14. TOD -1-2	Spread	winky winte
		
Observations of bacteria can be	done individually or in groups in the form of a	colonies. If the bBacteria is isolated into a solid
medium, then there is a group commonly re-	eferred to as a colony. The colony's shape is dif	ferent for each species and it is characteristic of
a particular species (Dwidjoseputro, 1981)	<u>.</u>	
The Selection Results Symbiont Bac	teria Producing Antibacterial Compour	ids
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	ITELATION IN THE PRESIDENT	
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Figure <u>1</u> 2. Symbiont bacterial isolates	(A1, A2, A3, A4, B1, B2, C1, C2) on a direct	challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>12</u> . Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>1</u> ⊋. Symbiont bacterial isolates	(A1, A2, A3, A4, B1, B2, C1, C2) on a direct	challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>12</u> . Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>12</u> . Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>12</u> . Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
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Figure <u>12</u> . Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>12</u> . Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>12</u> . Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
Figure <u>1</u> 2. Symbiont bacterial isolates		challenge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2)
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1 Figure <u>2</u> 3. Symbiont bacterial iso	et al a construction of the second se	
Tigure <u>2</u> 3. Symbiont bacterial is Based on the results of the direct	entry and the set of t	enge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2) 4 isolates tested showed inhibitory activity
Figure 23. Symbiont bacterial is Based on the results of the direct against <i>S.aureus</i> and only 2 of the 7 is	elates (D1, D2, D3, D4, E, F) on a direct challenge test, 7 bacterial isolates from 1 solates had inhibitory activity against <i>E</i> .	enge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2) 4 isolates tested showed inhibitory activity coli, The isolate codes that have inhibitory
Tipe 23. Symbiont bacterial is: Based on the results of the direct against <i>S. aureus</i> and only 2 of the 7 i zones against <i>S. aureus</i> bacteria are Th	entry and the second se	enge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2) 4 isolates tested showed inhibitory activity <i>voli</i> , The isolate codes that have inhibitory D2-D3-2, and TUD3-F-2, whereas TUD4-
Figure 23. Symbiont bacterial is Based on the results of the direct against <i>S. aureus</i> and only 2 of the 7 it zones against <i>S. aureus</i> bacteria are TT C1-2, And TUD4-C2-2 have showed in	2 2 2 2 2 2 2 3 2 3 2 3 2 3 2 3 2 3 2 3 2 3 3 3 3 3 3 3 3 4 5 4 5 5 1 3 1 1 3 1 1 1 1 1 1 1 1 1 1 1 1 1	enge test to <i>S.aureus</i> (1) and <i>E.coli</i> (2) 4 isolates tested showed inhibitory activity coli, The isolate codes that have inhibitory
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193 Bacterial isolates derived from the insidetissue showed have better activitybetter inhibition than bacterial_isolates 194 derived from the surfaceepibionts. Inhibitory zone and diameter measurement results against S.aureus and E.coli can be 195 seen in Figure 34 and Table 34. Positive controls have a broader spectrum in inhibiting both types of test bacteria with 16.8 196 197 mm inhibition against S.aureus and 13.8 mm against E.coli. Chloramphenicol with a concentration of 0.03 mg on a paper disc is highly active if its inhibition zone is more than 18 mm (Lay, 1994), while the dose of chloramphenicol (positive 198 199 control) used is lower at 0.01 mg, so it can be said that bacteria Test is sensitive to positive control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that a 200 201 supernatant still containing medium has no effect on the activity formed. From the stability of the measured inhibition zone, the antibacterial properties of the supernatant produced by the symbiotic bacteria act as inhibitors against Gram positive 201 202 203 204 205 bacteria and are merely bacteriostatic for Gram negative bacteria. Paper disc with a supernatant applied to a Gram positive bacterial plate indicates a stable clear zone even after a 48-hour incubation period. While against Gram negative bacteria, around the disc paper shows the presence of inhibitory activity but gradually become turbid before the incubation period reaches 24 hours.

According to Abubakar et al (2011) in Sartika (2014) the inner symbiotic bacteria generally have abundant populations and are specific microbes because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria originating from the surface have a population that is less suspected because it requires higher defense power to overcome the pathogens and predators that are around the algae.

Symbiont bacteria isolates in algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of the form of symbiotic mutualism. Algae provide the places and nutrients the bacteria need, while the bacteria encourage growth and protect the algal surface against pathogens (Hollants et al., 2012 in Sartika, 2014).

2066 2077 2088 2099 2100 2111 2122 213 214 215 216 217 218 229 220 221 2223 224 225 226 227 228 229 The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score for species level with a similarity of \geq 99% of the sequences present in GenBank, Then the species homology of the isolates tested was Lactobacillus plantarum. Classification of bacterial isolates are Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus plantarum.

Discussion

Antibacterial Potential Testing of Symbiont Bacteria Isolates by Discussion Paper Disc Applications

The antibacterial compounds produced by symbiont bacteria isolates showed different inhibitory activity against both tested bacteria S. aureus, and E. coli with clear zone observations around the paper dise. Inhibitory zone and diameter diameter measurement results against S.aureus and E.coli can be seen in Figure 4 and Table 4. According to Abubakar et al (2011) in Sartika et al (2014) the inner symbiotic bacteria generally have abundant populations and are specific microbes because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria originating from the surface have a population that is less suspected because it requires higher defense power to overcome the pathogens and predators that are around the algae. 230

231 Positive controls have a broader spectrum in inhibiting both types of test bacteria with 16.8 mm inhibition against S.aureus and 13.8 mm against E.coli. Chloramphenicol with a concentration of 0.03 232 mg on a paper dise is highly active if its inhibition zone is more than 18 mm (Lay, 1994), while the dose 233 234 of chloramphenicol (positive control) used is lower at 0.01 mg, so it can be said that bacteria Test is 235 sensitive to positive control. Negative control (NB without symbiotic bacterial inoculation) indicates 236 the absence of activity or inhibition zone, so it can be ascertained that a supernatant still containing 237 medium has no effect on the activity formed.



Figure 43. Results of antibiotic susceptibility test against S.aureus and E.coli

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From the stability of the measured inhibition zone, in general the antibacterial properties of the supernatant produced by the symbiotic bacteria act as bactericidal against Gram positive bacteria and are merely bacteriostatic in Gram negative. Paper disc with a supernatant applied to a Gram positive bacterial plate indicates a stable clear zone even after a 48 hour incubation period. While against Gram negative bacteria, around the disc paper shows the presence of inhibitory activity but gradually become turbid before the incubation period reaches 24 hours. Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Lay, 1994). Other factors that influence the ability of inhibitory inhibition are the concentration or intensity of antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the presence of organic matter and the degree of acidity (pH) (Sulistijowati and Mile, 2015).



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Table <u>3</u>4. Results of measurement of inhibitory zone diameter of antibacterial compounds

	Diameter of zone inhibition (mm)							
_	Gram positive			Gram negative			_	
Repetition	Symbiont bacterial (++)	Control (+)	Control (-)	Symbiont bacterial (++)	Control (+)	Control (-)	F	
.1	5.5	16	0	0	13.5	0	F	
2	7,8	17,5	0	0	14	0	\succ	
Average	6,7	16,8	0	0	13,8	0	F	
The area of t	he symptomatic s	upernatant inhibi	tion zone of Sa	ureus <mark>, is-</mark> was 6.7 m	m According to	Edrada (1998) in	F	
Kusumadewi (2004)							F	
and strong activity if							F	
supernatant obtained	l was still far from	the results of the	antibiotic activi	ty of the tested con	nparatochloramp	henicol r control.	F	
This is because the	antibacterial cor	npound of the a	applied extracted	symbiont bacter	ia is still<u>was</u> a	supernatant with		
thecontaining secon	dary metabolites.	it contains, but	However, the test	t results have indic	ated the presence	e of provide clear		
evidence of antibact	erial activity. Ger	nerally the chemi	ical structure of	metabolites from 1	marine products	is often different		
from the secondary	differs from those	se of terrestrial	origin metabolit	e of land_ (Gudbj	arnason 1999 in	Nofiani, 2005).		
Seawater contains an	n active inhibitor	agent for Gram	positive bacteria	, according to Oka	ami (1982) 2) in i	n Nofiani (2005)	F	
that seawater contain	ns an active inhibi	itor agent for org	anisms, seawate	r has the ability of	inhibitors a gain	st Gram positive		
bacteria,								
TTI di ta	c (1.1.1		11 0	1	a a		G	
	or sea water inni	bitor is not caus	ed by laga or si	alinity but because	there are antiba	acterial agents in	E	
seawater.								
Based on the	results of previous	s studies, most ba	eteria that live b	y associating with i	marine living cre	atures show great		
potential in_second	ary metabolite see	eretion with anti-	bacterial propert	ies (Burgesset et a	d., 1999; Armstr	ong et al., 2001;		
Yanet et al., 2003 in	Nofiani, 2005). <mark>S</mark>e	condary metabo	lites are not used	for growth and are	formed from pri	mary metabolites		
under stress conditi	ons. Examples o	f secondary met	abolites are ant	ibiotics, pigments	, toxins, ecologi	e and symbiotic	F	
competition effectso	ors, pheromones, o	enzyme inhibitor	s, immunomodu	ilating agents, anta	agonizing recept	ors and agonists,	C	
pesticides, antitumor	agents, and prom	noters of plant an	d animal growth	(Nofiani, 2005).				
Identification	n of Phenotype a	nd Genotype of	Symbiont Bact	eria				
							F	
Known characteristic	es of the microsof	nic identificatio	n and biochemic	al tests of symbio	at bacteria inclu	de the shape of a		
stem, non-acidic, no							U	
Based on the identif								
indicates there are								
Lactobacillus. Arcar			or naving simi	iai characters nan	iery <i>brocholititi</i> x	, Erystpetottirtx,	C	
Laciobacinus, Arcar	<i>юоистенит</i> , апа л	machnu.				<		
Based on phe	notypic identifica	tion results throu	igh cell staining	and biochemical to	esting, symbiont	bacteria haswere		

rod shaped, non acidic, non spore forming, non motile, aerobically grown, negative catalase, and positive to carbohydrate

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tests. In general, the identification of microscopically selected isolates showeds specific characteristics possessed by of	
lactic acid bacteria (<u>Lactobacillus, spp.</u>), such as round colonies, milky white, Gram positive with short stem cells, and doeswithout not formforming endospores (Desniar 2012 in Saskia, 2014). The genus Lactobacillus can be isolated from	Formatted: Font: 10 pt, Italic
several different habitats, eg from milkfish intestine (Sulistijowati and Mile, 2015), bekasam products (Ingratubun et al.,	Formatted: Font: 10 pt
2013), up to coastal mangrove waters (Yahya et al., 2014).	
The DNA of the symbiont bacteria isolates was amplified using primers 9F and 1541R. The DNA	
bands used were relevant to the resulting PCR product of about 1400 base pairs. The sequence of DNA	Commented [p23]: Moved to Materials and Methods
sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score	
for species level with a similarity of \geq 99% of the sequences present in GenBank, Then the species homology of the isolates tested was <i>Lactobacillus plantarum</i> . Classification of bacterial isolates are	
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus	
plantarum.	Commented [p24]: Moved to Results
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Lactobacillus plantarum_100%	Formatted: Left
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GTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAA/ CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGA	
GGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCT	A
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCG	
CATGTGTAGCGGTGAAATGCGTAGATATTGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCT	
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AGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGGAT ACGTCAAATCATCATGCCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGG	
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Antibacterials Potential Symbiont Bacteria of Brown Algae (Turbinaria conoides) Obtained fFrom Banten Bay **Serang District - Province Of Banten**Indonesian Waters

Niken Dharmayanti, Acf Permadi, Arma Anti, Resmi Rumenta Siregar Yuliati H Sipahutar, Resmi Rumenta Siregar, Arpan Nasri Siregar, Yuliati H Sipahutar, Aef Permadi, Randi Bokhi Salampessy, Sujulivani, Arpan Nasri Siregar, Randi Bokhi Salampessy, Sujuliyani, Siti Zachro Nurbani, Heni Budi PurnamasariArma Anti anti

Indonesia Study program-Program of Fish Processing Technology, Jakarta Technical University of Fisheries Technical University, Pasar Minggu 12520, South Jakarta, Indonesia Sekolah Tinggi Perikanan, Jakarta

ABSTRACT

Brown seaweed has the potential to produce bioactive compounds. It has been shown that the bacteria associated with seaweed are involved in the production of metabolites associated with their host. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes (Kalaivani et al., 2016). In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were brought from Lima Island, Serang District, Banten Bay. Symbiont bacteria withtested for antibacterial activity, were isolated using the bioassay test method. A total of 14 isolates were isolated, 6 of which came from the outside external tissue, while 8 isolates came from the inside of the algaeinternal tissue. Through the antagonistic test, 7 isolates showed inhibitory activity against <u>Staphy</u>lococcuS-aureus and 1 isolate bacteria showed the best-inhibition against both S.aureus and E.coli. Selected isolates have the ability to inhibit S.aureus after diffusion paper-disc tested._Phenotypic and genotypic identification showed that the species symbiont bacteria of Turbinaria conoides iswas Lactobacillus plantarum.

20 Keywords: bioassay, antagonistic, diffusion paper disc, Lactobacillus plantarum.

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1. INTRODUCTION

2 Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse and 3 unique compounds (Bahare S et al. 2019). Many substances obtained from seaweed, such as alginates, carrageenan, and agar 4 have been used for decades in traditional medicine, pharmacology, and food (Andrea GZ et al. 2019). Other compounds 5 have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory and antifouling activity. Therefore, seaweed can 6 provide promising bioactives that can be used in the treatment of human diseases, or new antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Much attention has been paid to developing 8 innovative projects for pharmaceuticals. seaweed applications, especially in the design of new antimicrobial drugs. Research 9 for the identification of promising algal species, standardization of analytical methods, isolation of compounds through 10 integrated fractionation of bioassays, detailed chemical characterization and evaluation of their safety, evaluation of 11 synergistic effects between components, and efforts to improve yields. and lowering extraction costs, is needed (Marie JP et 12 al. 2016).

13 Seaweed is an algae that lives in the sea and belongs to the division of thallophyta. The classification of seaweed 14 on pigment content consists of 4 classes, namely green seaweed (Chlorophyta), red seaweed (Rhodophyta), brown hacad 15 seaweed (Phaeophyta) and blond seaweed (Chrysophyta) (Suparmi and Sahri, 2009). Indonesia is the largest producer of 16 seaweed in the world (FAO 2016) cultured in nearshore coastal regions. In addition to its primary economical content, the 17 secondary metabolite content of seaweed has the <u>Seaweeds</u> potential of being a producerproduce of diverse bioactive 18 metabolites with vast activity as antibacterial, antiviral, antifungal and cytotoxic properties (Zainuddin and Malina, 2009 in 19 Siregar et al., 2012). Bacteria usually live on a host by performing a mutually beneficial symbiosis (Sahara et al., 2013). It 20 has been shown that the bacteria associated with seaweed as epiphytes or endophytes are involved in the production of 21 metabolites that together with their host. Microbes can be present as a living symbiotic in union with various marine algae 22 as epiphytes or endophytes_(Alessandro B et al. 2017Sartika et al. 2014, Kalaivani et al., 2016). <u>-Symbiont bacteria isolates</u> 23 in algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of the form of symbiotic 24 mutualism. Algae provide the places needed sites and nutrients the bacteria need, while the bacteria encourage growth and 25 protect the algal surface against pathogens (Mark LW et al. 2016Hollants et al., 2012 in Sartika et al. 2014). Seaweeds can 26 secrete secondary metabolites -with antibacterial properties-(Burgesset et al., 1999; Armstrong et al., 2001; Yanet et al., 27 2003 in Nofiani, 2005 (Emer S and Nissreen AG 2016). The recent scientific trends focus on search of phytochemicals from 28 marine algae due to their numerous health-promoting effects, including antioxidant, anti-inflammatory, antimicrobial, and 29 anti-cancer (Gupta et al. 2011)

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30 Tubunaria conoides belongs to the family of Sargassaceae (brown algae) is coming under the order of Fucales. It has 31 traditionally been used for children's fever, as a fertilizer, insect repellent, pesticide and antibacterialcidal (Arumugama P et 32 al. 2017)

33 34 Brown algae and other types have been extensively analyzed for their antibacterial and antifungal activity (Bhakuni 35 36 and Rawat, 2005). Previous research on brown algae Sargassum sp. Has been shown to have antimicrobial potential of bioactive proteins from bacteria symbiotic with it (Sartika et al. 2014) and the potential of bacterial algaeHere we evaluate 37 the properties of the brown algae Turbinaria conoides in producing bioactive compounds in inhibitingincluding the 38 inhibition of pathogenic bacteria Urinary Tract Infection (UTI)human pathogens (Kalaivani et al.-, 2016). <u>*T. conoides*</u> is a 39 tropical marine alga widely distributed in coastal waters in -SE Asia. -We chose this alga following extensive trials on other 40 common macroalgae including Sargassum spp. and Euchema cottoni. 41

The study focused on identification of macroalgae found in the sampling sites, isolation symbionts of the Turbinaria conoides, selection of symbiotic bacteria isolates, testing of antibacterial potency by diffusion of paper dises, and identification of the phenotype and genotype Turbinaria conoides symbiont bacteria.

48 Materials

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MATERIALS AND METHODS

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The materials used in this research are Turbinaria conoides,., pure cultures of S.aureus, pure culture of E.coli, aquadesh, nutrient broth (Oxoid), plate count agar (Oxoid), mueller hinton agar (Oxoid), sterile sea water, 70% alcohol, 95% alcohol, spirtus, crystal violet, iodine, safranin, immersion oil, carbolfuesin dyes, alcoholic acid, methylene blue, malachite green solution, safranin solution, filter paper, tissue, cotton, brown paper, paper disc, matches.

53 The equipments used are petri dishes, test tube, beaker, measuring cup, preparatory glass, measuring pipette 54 (omnipipette), dropper pipette, tip pipette, micro pipette, mortar, tube rack, scales (vibra), inoculation loops, Spatula, bent 55 glass, sterile plastic, magnetic stirrer, bunsen, hotplate (thermo scientific), scooter, microscope (olympus), scissors, tweezers, 56 autoclave (kemoto scientific), incubator (memmert), oven (memmert), shaker (thermo Scientific), refrigerator (selecta), 57 air flow (telstar), ohp markers, elastic bands, centrifuge (eppendorf), eppendorf tube, vortex mixer 58 (heidolph).Application GPS mobile phone 59

60 MethodsProcedures

61 Sampling

62 Samples of Turbinaria sp. (about 1 kg wet weight) was were taken from Lima island (S: --6.0010510; E: 106.153804) around 1 kg for determination in the morning around 7 at low tide allows the position of algae 1 meter below 63 64 the water on the sidelines of the reef directly dried in the bundle of the island. Sampling was continued at 16 o'clock when 65 the low tide and taked 500 grams for antibacterial test and kept in the plastic pouch and immediately filled with seawater, 66 with seaweed : water ratio of 1: 2 until submerged, when it already arrived on Serang filled with oxygen 1: 2 more air. The seweed stayed in the plastic with oxygen from Serang until Jakarta for a night and started done in the laboratorium in the 67 68 morning.Samples were maintained in fresh seawater for laboratory analyses within 24 hour of collection.

69 **Identification and Determination of Macroalga**

70 71 72 73 74 75 76 77 78 Dried Turbinaria sp. found in predetermined location or stations were recorded and identified by macroalgae type through algae base associated with observed macroalgae characteristics. The type of macroalgae used in this study was a genus of Turbinaria sp. The location is in the waters of the island of Lima, Banten Bay, Serang regency. The macroalgae determination used has done in LIPI Oceanography. Identification was performed on the method of form specification with reference to algae identification guidelines (Brigham et al., 2004; Lee, 2008)

Isolation of Symbiont Bacteria Producing Antibacterial Compounds

Surface of Algae: Epibionts were extracted from 15 grams of algae by rinsed rinsin with 30 mL of sterile sea water. The rinse water is was put into incubated in 30 mL of nutrient broth medium then shaken by shaker at room temperature for 24 hours. Inside of algae: as many asBioactive compound _15 grams of algae were rinsed with 30 mL of sterile sea water, were 79 80 extracted by erushed crushing 15 g of algafinely using mortal with a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension is then fedwas incubated into with 30 mL broth nutrient medium and shaken by shaker aatt room 81 temperature for 24 hours.

82 After extraction process, #the refreshed samples of in the 30 ml broth nutrient medium were diluted into 9 ml broth 83 nutrient sterile by 10⁻¹ up to 10⁻⁵. Each dilutions was were grown on a plate count agar medium by incubate them at 37 °C 84 for 2 x 24 hours. After incubating the petri dishes which were contain samples from each dilution, then the colonies bacteria 85 from alga would appear. The colonies bacteria producing antimicrobial compounds are were characterized by a clear zone

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86 around the coloniesy. Furthermore, the colonies with stable inhibition zones were collected -by and-isolating themted on 87 slant agar medium, with a clear code. 88 Commented [n4]: Is this after the incubat 89 Selection of Symbiont Bacteria Isolates Antagonistically against Pathogenic Bacteria 90 91 To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria, a qualitative test was 92 conducted directly by scratching or bottling round the isolates on the surface of the media that has been dispersed with test 93 bacteria (Eschericia -coli, and Staphylococcus-aureus). Then Media were incubated for 2 x 24-48 hours at 37 °C. Each 94 scratching round of isolates was then marked by its a unique code. 95 Inhibition zones were read as the point determined as those showing clear zones around the colony of simbiont* 96 bacteria isolates, the more clear zone of isolates in inhibit for both Escheriacia coli and Staphylococcus aureus are the better 97 their activity. Strains -that showed maximum antagonistic effect againsts tested pathogens were choosed and marked by its 98 eodeidentified. Isolates that These choosen isloate with appropriate code which was formed a clear zone or has with the a 99 highest activity are waswere isolated and selected for - further antibacterial testing by paper disc and identification of 100 <u>phenotype and genotype testin.g.</u> 101 Antibacterial Potential Testing of Symbiont Bacterial Isolate by Paper Disc Diffussion 102 Testing inhibitory the supernatant of symbiont bacteria on the for inhibitory growth of E.coli and S.aureus was 103 performed by the agar diffusion method (Hudzicki, 2009Grela E et al. 2018) REFERENCE), Supernatant was obtained by 104 separating the filtrate and supernatant by centrifugation processcentrifuge for 1 hour, temperature at (25 °C and 3000 rpm). 105 Paper discs containing supernatant 40 µL and the negative control nutrient broth 40 µL which has allowed were dried left 106 for 1 hour to reduce the water excess-(dried), negative control nutrient broth 40 µL also has allowed for 1 hour to reduce 107 the water excess (dried) and positive control chloramphenicol 0.01 mg/mL, were placed on the surface of the Mueller Hinton 108 Agar A-medium containing 1 mL test bacteria. Furthermore and incubated for 2 x 2448 hours at 37 °C. The supernatant 109 diffuses from the disc into the agarr in decreasing amounts the further it is away from the dise. If the organism is killed or 110 inhibited by both the supernatant and chloramphenicol as antibiotic positive control, there will be no growth in the immediate 111 112 113 area around the disc, this is called the zone of inhibition. The zone sizes awere compared up on a standardized to give a result of to assess bioactivity as sensitive, resistant, or intermediate, te then It was observed and measured its iIn each case the resistance zone -where shows no colonies growth with by a ruler was measured by using ruler to the nearest mm 114 115 116 117 118 119 120 121 122 123 124 125 Identification of Phenotype and Genotype of Symbiont Bacteria In general, General bacterial identification was performed_ in accordance with the microbial analysis procedure in the laboratory (Phumudzo T, 2013Lay, 1994 and identification keys from Cowan and Steel (1993)) by performing followed colony characteristic observations on liquid medium and solid medium, observing cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and test Biochemistry test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase). The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria conoides samples on the agar medium in pour plating. Observation of medium incubated with temperature 37°C was done at incubation time reached 24 hours and 48 hours. The data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from the Turbinaria conoides-seaweed. Determination of the type of bacteria was performed based on-identification keys from Cowan and Steel (1993). Symbiont bacteria species was determined by molecular testing. 126 127 128 129 130 131 The DNA of the symbiont bacteria isolates was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs, -The PCR reaction used a PCR machine (Eppendorf German)

with a first predenaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C for 30 seconds, primary attachment at 50 ° C for 30 seconds and extension at 72 ° C for 90 seconds. After 30 cycles completed, followed by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular identification was done 132 133 through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989. Modified). PCR Amplification on 16S rDNA using Primer 9 F: 5' -- AAG GAG GTG ATC CAG CC-3' and Primer 1541 134 135 R: 5' - GAG TTT GAT CCT GGC TCA G - 3' (White et al., 1990; O'Donnell, 1993). The analysis of nitrogen base sequence readings using was performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied 135 136 137 The next sequenced raw data waswere trimmed and assembled using the BioEdit program Biosystems). (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data that has beenwere assembled in BLAST with genomic 138 data that has been registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) 139

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140 RESULTS RESULTS AND DISCUSSION 141 Formatted: Font: 10 pt 3. 142 Formatted: Indent: Left: 0,75 cm, No bullets or numbering The Result of Identification and Determination of Macroalga 143 144 The macroalgae observation area and the sample site obtained are determined based on the location coordinate point. The location of macroalgae observation S: -6.0010510; E: 106.1538040 has morfology characteristic as Cylindrical 145 146 rods, creet, rough, there are traces of branching. Holdfast is a small disc with radial expansion root. The branches rotate around the main trunk. Leaves are unity consisting of stalks and sheets . It named Turbinaria conoides. 147 Formatted: Font: (Default) Times New Roman, 10 pt 148 149 Turbinaria conoides 150 151 The Result of Symbiont Bacteria Isolation 152 The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria 153 conoides samples on the agar medium in pour platting. Observation of medium incubated with temperature 37°C was done 154 at incubation time reached 24 hours and 48 hours. When incubated, the individual microbial cells multiply so rapidly that 155 within 18 to 24 hours a visible mass of cells is formed and is called a colony (Pelzcar and Chan, 1986). 156 Formatted: Font: (Default) Times New Roman, 10 pt Formatted: Justified 157 158 Figure 1. Growth of symbiont bacteria on agar medium 159 The grown From 40 s Samples consisted of the inside and outside of the algae, as many as 20 petri for 2 replications Commented [p18]: Is this the number of samples tested? 160 resulted in colonies with the inhibit zone of 14 colonies, 6 of which were from the outside epibionts, while the other 8 came 161 162 from the inside of the algaealgal tissue. The results of identification of colonies grown on mixed cultures can be seen in Table 21.- and identification of isolates isolated into slant agar can be seen in Table 23. 163 164 165 166 167 168 169 170 171 Formatted: Font: 10 pt 172 Formatted: Font: 9 pt 173 Tabel 21, Macroscopic forms of bacterial colonies Morphology of colonies Formatted: Font: 9 pt No Colony code Shape Color Edges Elevation Formatted Table

						////
1	TUL ² -A1-2	Round	White	Flat	Convex shiny	////
2	TUL ² -A2-2	Round	White	Flat	Convex shiny	////.
3	TUL ² -A3-2	Round	White	Flat	Convex shiny	
4	TUL ² -A4-2	Round	White	Flat	Convex shiny	
5	TUL ² -B1-2	Round	White	Crooked	Convex shiny	
6	TUL ² -B2-2	Round	White	Crooked	Convex shiny	////
7	TUD ⁴ -C1-2	Round	White	Flat	Convex shiny	
8	TUD4-C2-2	Round	White	Flat	Convex shiny	
9	TUD ² -D1-2	Round	White	Crooked	Convex shiny	
10	TUD ² -D2-2	Round	White	Crooked	Convex shiny	
11	TUD ² -D3-2	Round	White	Crooked	Convex shiny	
12	TUD ² -D4-2	Round	White	Crooked	Convex shiny	
13	TUD ⁵ -E-2	Round	White	Flat	Convex shiny	
14	TUD ³ -F-2	Round	White	Flat	Convex shiny	

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 If a TOD-F-2
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 **** The code of number 2 identifies the isolate obtained from the second repeat

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182 Table <u>32</u> Identification of the isolates on slant agar

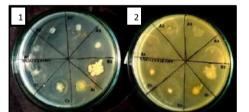
N.	Code of isolates	Solid medium		
No	Code of isolates	Shape	Color	
1.	TUL ² -A1-2	Spread	Milky white 🖌	
2.	TUL ² -A2-2	Spread	Milky white	
3.	TUL ² -A3-2	Spread	Milky white	
4.	TUL ² -A4-2	Spread	Milky white	
5.	TUL ² -B1-2	Rhizoidal	Cloudy white	
6.	TUL ² -B2-2	Rhizoidal	Cloudy white	
7.	TUD ⁴ -C1-2	Spread	Milky white	
8.	TUD ⁴ -C2-2	Spread	Milky white	
9.	TUD ² -D1-2	Rhizoidal	Cloudy white	
10.	TUD ² -D2-2	Rhizoidal	Cloudy white	
11.	TUD ² -D3-2	Rhizoidal	Cloudy white	
12.	TUD ² -D4-2	Rhizoidal	Cloudy white	
	TUD5-E-2	Spread	Milky white	
	TUD ³ -F-2	Spread	Milky white	

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184 185 186 Observations of bacteria can be done individually or in groups in the form of colonies. If the bBacteria is isolated into a solid medium, then there is a group commonly referred to as a colony. The colony's shape is different for each species and it is characteristic of a particular species (Erin RSDwidjoseputro, 19812012).

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189 190 The Selection Results Symbiont Bacteria Producing Antibacterial Compounds



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Figure 12. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to S.aureus (1) and E.coli (2)

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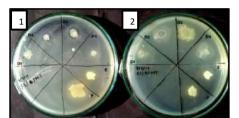


Figure 23. Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to S.aureus (1) and E.coli (2)

Based on the results of the direct challenge test, 7 bacterial isolates from 14 isolates tested showed inhibitory activity against *S. aureus* and only 2 of the 7 isolates had inhibitory activity against *E. coli*. The isolate codes that have inhibitory zones against *S. aureus* bacteria are TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2, whereas TUD4-C1-2, And TUD4-C2-2 have showed inhibition zones against both pathogenic bacteria being tested but the inhibitory activity against *E. coli* is was not as good as its inhibition against *S. aureus*.

Symbiotyc bacterial isolates with a specific code that has a resistor zone are re-selected by looking at the best and largest clear zone. From the observation result, it was determined that isolates with code TUD4 C2-2 were isolates which had the best inhibition zone. Based on the code given, it is known that this isolate was obtained from the algae's inner sample, at 10-4 dilution, the second colony of the isolated third plate, and a colony obtained in the second repetition. Isolates with a specific code that has a showing inhibition zone_were re-selected by looking at the best and largest clear zone. Isolates with code TUD4-C2-2 were isolates which had the best inhibition zone. From the observation result, it was determined that isolates with code TUD4-C2-2 were isolates which had the best inhibition zone. Based on the code given, it is known that this isolate was obtained from the algae's inner sample, at 10⁻⁴ dilution, the second colony of the isolated third plate, and a colony obtained in the second repetition.

Bacterial isolates derived from the insidetissue showed have better activitybetter inhibition than bacterial_isolates
derived from the surfaceepibionts. Inhibitory zone and diameter -measurement results against S. aureus and E. coli can be seen in Figure 34 and Table 34. Positive controls have a broader spectrum in inhibiting both types of test bacteria with 16.8 mm inhibition against *S. aureus* and 13.8 mm against *E. coli*. Chloramphenicol with a concentration of 0.03 mg on a paper
disc is highly active if its inhibition zone is more than 18 mm (Mounyr B et al. Lay, 19942016), while the dose of chloramphenicol (positive control) used is lower at 0.01 mg, so it can be said that bacteria Test is sensitive to positive control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that a supernatant still containing medium has no effect on the activity formed. From the stability of the measured inhibition zone, the antibacterial properties of the supernatant produced by the symbiotic bacteria act as inhibitors
against Gram positive bacteria and are merely bacteriostatic for Gram negative bacteria. Paper disc with a supernatant applied to a Gram positive bacterial plate indicates a stable clear zone even after a 48-hour incubation period. While against Gram negative bacteria, around the disc paper shows the presence of inhibitory activity but gradually become turbid before the incubation period reaches 24 hours.

According to Abubakar et al (2011) in Sartika (2014) the inner symbiotic bacteria generally have abundant populations and are specific microbes because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria originating from the surface have a population that is less suspected because it requires higher defense power to overcome the pathogens and predators that are around the algae.

Symbiont bacteria isolates in algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of the form of symbiotic mutualism. Algae provide the places and nutrients the bacteria need, while the bacteria encourage growth and protect the algal surface against pathogens (Hollants et al., 2012 in Sartika, 2014).

The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score for species level with a similarity of \geq 99% of the sequences present in GenBank, Then the species homology of the isolates tested was *Lactobacillus plantarum*. Classification of bacterial isolates are *Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacilluscae; Lactobacillus; Lactobacillus plantarum*.

37 <u>Discussion</u>

Antibacterial Potential Testing of Symbiont Bacteria Isolates by Discussion Paper Disc
 Applications

The antibacterial compounds produced by symbiont bacteria isolates showed different inhibitory activity against both
 tested bacteria *S. aureus* and *E. coli* with clear zone observations around the paper disc. Inhibitory zone and diameter diameter
 measurement results against *S. aureus* and *E. coli* can be seen in Figure 4 and Table 4. <u>According to Irma ESM Abubakar et</u>
 al (2011) in Sartika et al (2014) the inner symbiotic bacteria generally have abundant populations and are specific microbes

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TGGTCAGGACGAGACGCGATGGCTGACACGGGAGACCTGGCCAGAGGGGGATAACACCGGGGGGATAACACCCGGAGAGTGCTAATACCG CATAACAACTTGGACGCATGGTCAGGCATGGAAGAGTGCCTGGCGAGAGGGTAATCGGCGCGGGGATAACACCGGGGGGATGCAAATCACG GTGGGGAACGGGCAGCAGGCAGGCAGGCGGCAGCGCGGCGCGTGTCGGCACACGCGCGGGGATAACACGCCCCAAA CTCCTACGGGAGGCAGCAGGGAATCTTCCACAATGGACGAAAGTCGTAGGAGCAACGCCGCGGTGAGAGCACGGCCCAAA CTCCTACGGGAGGCAGCGGGGAATACGTAGGTGGGCAAGCGTTGTCAGGATATTGACGGTATTTAACCAGAAAGGGCCACGGGCTA ACTACGTGCCAGGCAGCCGGGGTAATACGTAGGTGGCAACGGCGAGGTATTTAACGAAAGGGACAGTGGAAACCC GACAGGCGGTGGAAATGCGTAGATGATGGGGAAGCGTGCGGAGCGCTGTCGGAACGCGAGGGGCGTTTTTA AGTCTGATGCGGAGAATGCGTAGATGATGGTGGGAAGCGCCAGGGGGAGGCGGCTGTCGGAACGCAGGGGCGCT CAGTGCTACGGGTAGAAAGGATTAAGCTAGGAGAAGTGCATACGGGAAACTGGGAAACTGGCAAAGGGACAGTGGAACTC GAAAGTATGGGTAGAAAAGGATTAAGCTAGGCGAGGAGCGCGCGAGGAGGGGGGCTGTCGGAACGCAGGGGGCCC CACAAGCGGTGGAAACGGATTAAGTACCTGGGAAACCCCAGGGCGCGGGGAGAGGGGGGTTGCGGAAGCGCCAAGGGGCCCC CACAAGCGGTGGAACAGGATTAAGCTACCCGGGAGAACGCCCCGCAAGGCCGGGAGAAGTGGTAAGGGGTTACGGCGCCCC TCAGTGCTTGCGGGGACATGGGTTGACGCCGGGGGCACGCGCGCG	bands used were relevant to the resulting PCR product of about 1400 base pairs. The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score for species level with a similarity of \geq 99% of the sequences present in GenBank, Then the species aboutlogy of the isolates tested was <i>Lactobacillus plantarum</i> . Classification of bacterial isolates are <i>Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus plantarum</i> .	Commented [p27]: Moved to Results
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GGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGGGAGTAACTGTTCAGGTATTTGACGGTATTTAACCAGAAAGCCACGGCTA ACTACGTGCCAGCAGCCGCGGGTAATACGTAGGTGGCAAGCGTGTCTCGGAAACTGGGAAACTGGGAAACTGGGAACGCGAGGCGCGTTTTTT AAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAGTGCATCGGAAACTGGGAAACTGGGAAACTGGCAAAGGGGCACGTGGAACTC GAAAGTATGGGTAGCAAACAGGATTATGGAAGAACGCCAGGGGGGGCTGTCGGACGCGGCTGTGTGACGCAGGGCCCC GAAAGTAGGGTAGCAACAGGATTAAGCATCCGGTGGGAGTACCGCCCAAGGGCTGTCGGACGTGTGGAGGGTTCCGCCCT TCAGTGCTGCAGCTAACGCATTAAGCATCCGGCGAGGACGCCGCAAGGCCGGCTGTGAAGTGTTGGAGGGCTCC GACAAGCGGTGGAACAGGATTAAGCATCCGGCGAGGACGCCGCAAGGCCGGAGAACTCAAAGGAATTGACGGGGGCCCC CACAAGCGGTGGAGCATGGGTTTAATTCGAAGCTACGCCGAAGACCTTACCAGGCCTGGAACTCCAAAGGAATTA GACGTTCACTTCGGGACATGGGTGCATGGTGTGCGTGCGCGGAGGACCTGCCGGTGCAGAACTCGCAAGGCGAAGGCGGGGATG ACGCCAAACCCTTATTATCGAGTGGGCACGGCTGCCGGCCCGGCCCGTGCAGAACTCCGGAGGAAGGTGGGGATG CTAATCCTTAAGCCCTTATGACCTGGGCTACACCGGCGCCACGCCCGTGCAAACCCGAAGGCGAGGAAGGTGGGGATC CTAATCCTTTAAGCCCTTGCGGGTGACACCCGTGCTACACCAGGGGTACGGCAACTCGCGAAGCTCGCGAGGAAGGT CTAATCGTTCAAGCCCTTGGGGTGACACCCGTGCTACACCAGGGGTACGGCGAACTCCCGGAGGAAGGT ATGCCGCGGGTGAATACGTTCCCGGGCCTTGTACGCCGCCGCCCGC	bands used were relevant to the resulting PCR product of about 1400 base pairs. [The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score for species level with a similarity of ≥ 99% of the sequences present in GenBank, Then the species nomology of the isolates tested was <i>Lactobacillus plantarum</i> . Classification of bacterial isolates are <i>Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus</i> <i>plantarum</i> .]	Commented [p27]: Moved to Results Formatted: Font: (Default) Arial, Bold
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AGCGCAACCCTTATTATCAGTIGCCAGCATTAGTIGGGCACTCGGGTGGGAACTGCCGGGTGACAAACCGGAGGAAGGTGGGGATG ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAACGGGTGGGATGGTACAACGAGGTGGGAACTCGCGAGAGAAGG CTAATCCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCCAACTGGCCTACATGAACGGAGTGGGAACTCGCGGAGGAGGAGG ATGCCGCGGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACGAGGAGTTTGTAACACCCCAAAGTC Formatted: Font: 9 pt	bands used were relevant to the resulting PCR product of about 1400 base pairs. The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score for species level with a similarity of ≥ 99% of the sequences present in GenBank, Then the species nomology of the isolates tested was Lactobacillus plantarum. Classification of bacterial isolates are Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus plantarum. Lactobacillus plantarum_100% GCTCAGGACGAACGCTGGCGAGCGTGCCTAATACATGCAAGTCGAACGAA	Commented [p27]: Moved to Results Formatted: Font: (Default) Arial, Bold
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ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCGTCACACCATGAGAGTTTGTAACACCCCAAAGTC Formatted: Font: 9 pt	bands used were relevant to the resulting PCR product of about 1400 base pairs. [The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score for species level with a similarity of ≥ 99% of the sequences present in GenBank, Then the species homology of the isolates tested was <i>Lactobacillus plantarum</i> . Classification of bacterial isolates are <i>Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus</i> <i>plantarum</i> .]	Commented [p27]: Moved to Results Formatted: Font: (Default) Arial, Bold
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Figure 4. Sequens of 16S rDNA Formatted: Font: 9 pt	bands used were relevant to the resulting PCR product of about 1400 base pairs. The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate score for species level with a similarity of ≥ 99% of the sequences present in GenBank, Then the species bomology of the isolates tested was Lactobacillus plantarum. Classification of bacterial isolates are Bacteria; Firmicutes; Bacilli; Lactobacillus; Lactobacillus; Lactobacillus; blantarum. CCCAGGACGACGCTGGCGACGCGTGCCTAATACACGCAGGAGCGGACGACGGGGGATAACACCTGGAAACAGATGCTAATACCAG CCCAGGACGAACCGCTGGAACTGGTGACTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCAG CATAACACTTGGACGACGCTGGCGACGTGCCTAATACATGCAAGTGGCCCAGGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCAG CCCAGGACGACACGCTGGCGACGTGGCAACCGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCAG CTCAGGGACAGCGCTGGCGACACTGGCGAAGCGTGGGAAACCTGGCCAGAGGGGAAACACCTGGGACAACGGCCCCAAA CTCCAGGGGAACGGCGGGGAATACGTGAGAGACACTGGCCAAGGCGCGCGTGTTTAACCAGAAAGGGACCCAAAA CTCATACGGGAAGCAGCATGGGAAACACACGATGGGCAAACGGCGCGGTATTAACCAGAAAGGACCCGAGCGCGGTTTTAACCAGAAAACACGGGACGCGGGGTTTCCAGGAAACCAGGCGCGGCGTTTAACCAGAAAGGAACCACGGCGGGGTTTTAACCAGAAAGGAACACCGGAGGAAACTGGGCAAACTGGGAAACTGAAGGAAG	Commented [p27]: Moved to Results Formatted: Font: (Default) Arial, Bold
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k27 Genotypic identification of symbiont bacteria were using DNA encoding the 16S rDNA gene to determine genus and 328 strain. 16S rDNA can be used as a molecular marker for species definition because this molecule exists in every organism 329 with a function identical to all organisms.- Data offor base sequence encoding gene of 16S rDNA can be seen in figure 4, it 330 331 shows that symbiont bacteria has accurate scores for species levels with a similarity 100% of the sequences present in GenBank (Figure 4), The species homology of the tested isolate was Lactobacillus plantarum.

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CONCLUSION

332 Based on the survey results revealed that macroalgae were found in the Gulf of Banten, Serang district, Province of 333 334 Banten, named Turbinaria conoides. The result of the isolation of bacterial symbionts Turbinaria conoides isolates obtained 335 from colonies that produce inhibition zone in mix culture are 14 isolates, six of which came from the outside, while eight 336 other isolates came from the inside of the algae. Selection of 14 isolates through qualitative antagonist test showed that 7 337 isolates showed inhibitory activity against S.aureus and 2 isolates showed the best inhibition against E.coli. In general, 338 isolates with code TUD4 C2-2 were selected isolates and showed a better potential for S. aureus through diffusion test of 339 paper disc. Through molecular (DNA) test it was known that the symbiont species of Turbinaria conoides was Lactobacillus 340 plantarum. 341

Turbinaria conoides, is one of macroalga which is find atcommonly found in the gulf of Banten, Serang district, province of Banten. Based on the results of this This research known shows that symbiont bacteria Lactobacillus plantarum, eould living in the macroalga as endophyticare endophytic and potentially useful as an antibacterial agent against common pathogens.- The symbiont bacteria produced bioactive compound which was inhibited Gram positif phatogen bacteria Staphylococcus aureus.

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1 Antibacterials potential of symbiont bacteria of brown algae (*Turbinaria conoides*) obtained from Indonesian waters

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Abstract. Brown seaweeds haves the potential to produce bioactive compounds. It has been shown that the bacteria associated with seaweeds are involved in the production of metabolites associated with their host. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes. In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were tested for antibacterial activity. A total of 14 isolates were isolatedfound, 6 of which came from external tissue, while 8 isolates-came from internal tissue. Through the antagonistic test, 7 isolates showed inhibitory activity against *Staphylococcuareus Staphylococcus* and *E.coli*. Phenotypic and genotypic identification showed that the species-symbiont bacteria species was *Lactobacillus plantarum*.

14 Keywords: bioassay, antagonistic, diffusion paper disc, Lactobacillus plantarum

INTRODUCTION

16 Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse, and unique 17 compounds (Bahare S et al. 2019). Many are the substances obtained from seaweed, such as alginates, carrageenan, and 18 agar, which have been used for decades in traditional medicine, pharmacology, and food (Andrea GZ et al. 2019). Other 19 compounds have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory, and antifouling activity. 20 Therefore, seaweed can provide promising bioactive that can be used in the treatment of human diseases or new 21 antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Much attention has 22 been paid to developing innovative projects for pharmaceuticals. Seaweed applications, especially in the design of new 23 antimicrobial drugs. Research for the identification of promising algal species, standardization of analytical methods, 24 isolation of compounds through integrated fractionation of bioassays, detailed chemical characterization and evaluation of 25 their safety, evaluation of synergistic effects between components, and efforts to improve yields. and lowering extraction 26 costs is needed (Marie JP et al. 2016).

27 It has been shown that the bacteribacteria associated with seaweed as epiphytes or endophytes are involved within the 28 assembly of metabolites (Alessandro B et al. 2017). It's traditionally been used for children's fever, as a fertilizer, 29 repellantrepellent, including antioxidant, anti-inflammatory, antimicrobial, and anti-cancer (Gupta et al. 2011) Turbinaria 30 conoides belongs to the family of 17the recent scientific trends target the pursuit for phytochemicals from marine algae du 31 to their numerous health-promoting effects, pathogens (Mark LW et al. 2016). Seaweeds can secrete secondary metabolite 32 with antibacterial properties (Emer S and Nissreen AG 2016). The form of symbiotic mutualism. Algae provide needed 33 sites and nutrients, while the bacteria encourage growth and protect the algal surface against symbiont bacteria isolates in 34 algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a results of infections acquired 35 from the community (Arumugama P et al. 2017) It here we evaluate the properties of the brown alga Turbinaria conoides 36 in producing bioactive compounds including the inhibition of human pathogens (Kalaivani et al. 2016). T. conoides is a 37 tropical marine algae widely distributed in coastal waters in Asia. We chose this algae following extensive trials on other 38 common macroalgae including Sargassum spp. and Euchema cottoni.

39

15

MATERIALS AND METHODS

40 Procedures

41 Sampling

42 Samples of *Turbinaria sp.* (about 1 kg wet weight) were taken from Lima island (S: -6.001051; E: 106.153804) 43 Samples were maintained in fresh seawater for laboratory analyses within 24 hours of collection.

44 Isolation of symbiont bacteria producing antibacterial compounds

45 Epibionts were extracted from 15 grams of algae by rinsin with 30 mL of sterile sea water. The rinse water was 46 incubated in 30 mL of nutrient broth medium shaken at room temperature for 24 hours. Bioactive compound was Formatted: Font: Not Bold, Italic

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extracted by crushing 15 g of algawith a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension
 was incubated with 30 mL broth nutrient medium and shaken att room temperature for 24 hours.

49 After extraction process, the refreshed samples in the 30 ml broth nutrient medium were diluted into 9 ml broth nutrient 50 sterile 10^{-1} up to 10^{-5} . Each dilutions were grown on a plate count agar medium by incubate them at 37 °C for 2 x 24 hours. 51 After incubating the petri dishes which were contain samples from each dilution, then the colonies bacteria from alga 52 would appear. The colonies bacteria producing antimicrobial compounds were characterized by a clear zone around the 53 colonies. Furthermore, the colonies with stable inhibition zones were collected by isolating them on slant agar medium, 54 with a clear code.

55 Selection of symbiont bacteria isolates antagonistically against pathogenic bacteria

To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria, a qualitative test was conducted directly by scratching round the isolates on the surface of the media that has been dispersed with test bacteria (*Eschericia coli* and *Staphylococcus aureus*)(ref). Media were incubated for 48 hours at 37 °C. Each scratching round of isolates was then marked by a unique code.

Inhibition zones were determined as those showing clear zones around the colony of symbiont bacteria isolates, for both *Escheria coli* and *Staphylococcus aureus*. Strains that showed maximum antagonistic effect against tested pathogens were identified. These were isolated and selected for further antibacterial testing by paper disc and identification of phenotype and genotype.

64 Antibacterial potential testing of symbiont bacterial isolate by paper disc diffusion

65 Testing the supernatant of symbiont bacteria for inhibitory growth of E.coli and S.aureus was performed by the agar diffusion method (Grela E et al. 2018)). The supernatant was obtained by separating the filtrate and supernatant by 66 67 centrifuge for 1 hour (25 °C and 3000 rpm). Paper discs containing supernatant 40 µL and the negative control nutrient 68 broth 40 µL were left for 1 hour to reduce the water excess, and positive control chloramphenicol 0.01 mg/mL, were 69 placed on the surface of the Mueller Hinton Agar medium containing 1 mL test bacteria and incubated for 48 hours at 37 70 C. The supernatant diffuses from the disc into the agar. If the organism is killed or inhibited by both the supernatant and 71 chloramphenicol as an antibiotic positive control, there will be no growth in the immediate area around the disc, this is 72 called the zone of inhibition. The zone sizes were compared to assess bioactivity as sensitive, resistant, or intermediate, in 73 each case the resistance zone where shows no colonies growth was measured by using a ruler to the nearest mm.

74 Identification of phenotype and genotype of symbiont bacteria

75 General bacterial identification (Phumudzo T, 2013) followed colony characteristic observations on liquid medium and 76 solid medium, observing cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and Biochemistry 77 test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase). The initial selection of isolates from mixed 78 cultures was carried out after enrichment and planting of Turbinaria conoides samples on the agar medium in pour plating. 79 Observation of medium incubated with temperature 37°C was done at incubation time reached 24 hours and 48 hours. The 80 data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from 81 Turbinaria conoides. Determination of the type of bacteria was performed based on ???. Symbiont bacteria species were 82 determined by molecular testing.

83 The DNA of the symbiont bacteria isolateds was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf 84 German) with a first pre-denaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C 85 for 30 seconds, primary attachment at 50 ° C for 30 seconds, and extension at 72 ° C for 90 seconds. After 30 cycles 86 completed, Ffollowed by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular 87 88 identification was done through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989. Modified). PCR Amplification on 16S rDNA using Primer 9 F: 5 -- AAG GAG GTG ATC 89 CAG CC-3' and Primer 1541 R: 5' - GAG TTT GAT CCT GGC TCA G - 3' (White et al., 1990, O'Donnell, 1993). The 90 91 analysis of nitrogen base sequence readings was performed with an automated DNA sequencer (ABI PRISM 3130 Genetic 92 Analyzer) (Applied Biosystems). The next sequenced raw data were trimmed and assembled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data 93 registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) 94

RESULTS AND DISCUSSION

96 The Result of Symbiont Bacteria Isolation

95

97 Samples consisted of the inside and outside of the algae, as many as 20 petri for 2 replications resulted in colonies with 98 the inhibit zone of 14 colonies, 6 of which were from epibionts, while the other 8 came from the algal tissue. The results 99 of identification of colonies grown on mixed cultures can be seen in Table 1. and identification of isolates isolated into 910 slant agar can be seen in Table 2. **Commented [A1]:** Please incert a reference paper for this procedure???

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101 Tabel 1. Macroscopic forms of bacterial colonies

Colony code			Morphology of c	olonies
Colony code	Shape	Color	Edges	Elevation
TUL ² -A1-2	Round	White	Flat	Convex shiny
TUL ² -A2-2	Round	White	Flat	Convex shiny
TUL ² -A3-2	Round	White	Flat	Convex shiny
TUL ² -A4-2	Round	White	Flat	Convex shiny
TUL ² -B1-2	Round	White	Crooked	Convex shiny
TUL ² -B2-2	Round	White	Crooked	Convex shiny
TUD4-C1-2	Round	White	Flat	Convex shiny
TUD4-C2-2	Round	White	Flat	Convex shiny
TUD ² -D1-2	Round	White	Crooked	Convex shiny
TUD ² -D2-2	Round	White	Crooked	Convex shiny
TUD ² -D3-2	Round	White	Crooked	Convex shiny
TUD ² -D4-2	Round	White	Crooked	Convex shiny
TUD ⁵ -E-2	Round	White	Flat	Convex shiny
TUD ³ -F-2	Round	White	Flat	Convex shiny

102 103

Information: *The code of isolates TUL/TUD states the isolates originating from the outer/inner algae

104

** The code of isolates (2), (3), (3) states isolates obtained from the duilution *** The code of isolates (2), (3), (3) states isolates obtained from the duilution *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the 105 106 107 number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed on the plate

108 **** The code of number 2 identifies the isolate obtained from the second repeat

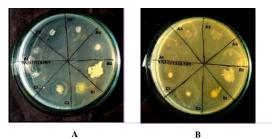
109 Table 2. Identification of the isolates on slant agar

Code of isolates	Solid medium		
Code of isolates	Shape	Color	
TUL ² -A1-2	Spread	Milky white	
TUL ² -A2-2	Spread	Milky white	
TUL ² -A3-2	Spread	Milky white	
TUL ² -A4-2	Spread	Milky white	
TUL ² -B1-2	Rhizoidal	Cloudy white	
TUL ² -B2-2	Rhizoidal	Cloudy white	
TUD4-C1-2	Spread	Milky white	
TUD ⁴ -C2-2	Spread	Milky white	
TUD ² -D1-2	Rhizoidal	Cloudy white	
TUD ² -D2-2	Rhizoidal	Cloudy white	
TUD ² -D3-2	Rhizoidal	Cloudy white	
TUD ² -D4-2	Rhizoidal	Cloudy white	
TUD ⁵ -E-2	Spread	Milky white	
TUD ³ -F-2	Spread	Milky white	

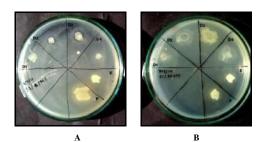
110 Bacteria is isolated into a solid medium, then there is a group commonly referred to as a colony. The colony's shape is

111 different for each species and it is characteristic of a particular species (Erin RS 2012).

112 The Selection Results Symbiont Bacteria Producing Antibacterial Compounds



113 Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to S.aureus (A) and E.coli (B) **Commented [A3]:** The images are of poor quality, I would like to see in more detail these results, to determine real inhibition.



114 **Figure 2.** Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to *S.aureus* (A) and *E.coli* (B)

Based on the results of the direct challenge test, 7 bacterial isolates from 14 isolates tested showed inhibitory activity against *S.aureus* and only 2 of the 7 isolates had inhibitory activity against *E.coli*. The isolate codes that have inhibitory zones against *S.aureus* bacteria are TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2, whereas TUD4-C1-2, And TUD4-C2-2 showed inhibition zones against both pathogenic bacteria being tested but the inhibitory activity against *E.coli* was not as good as its inhibition against *S.aureus*.

Isolates with showing inhibition were re-selected by looking at the best and largest clear zone. Isolates with code 120 121 TUD4-C2-2 were isolates which had the best inhibition zone. Bacterial isolates derived from tissue showed better 122 inhibition than isolates derived from epibionts. Inhibitory zone and diameter measurement results against S.aureus and 123 E.coli can be seen in Figure 3 and Table 3. Positive controls have a broader spectrum in inhibiting both types of test bacteria with 16.8 mm inhibition against S.aureus and 13.8 mm against E.coli. Chloramphenicol with a concentration of 124 125 0.03 mg on a paper disc is highly active if its inhibition zone is more than 18 mm (Mounyr B et al., 2016), while the dose 126 of chloramphenicol (positive control) used is lower at 0.01 mg, so it can be said that bacteria Test is sensitive to positive 127 control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that a supernatant still containing medium has no effect on the activity formed. From the stability 128 129 of the measured inhibition zone, the antibacterial properties of the supernatant produced by the symbiotic bacteria act as 130 inhibitors against Gram positive bacteria and are merely bacteriostatic for Gram negative bacteria. Paper disc with a 131 supernatant applied to a Gram positive bacterial plate indicates a stable clear zone even after a 48-hour incubation period. 132 While against Gram negative bacteria, around the disc paper shows the presence of inhibitory activity but gradually become turbid before the incubation period reaches 24 hours. 133

The antibacterial compounds produced by symbiont bacteria isolates showed different inhibitory activity against both tested bacteria *S.aureus* and *E.coli*. According to Irma ESM (2011) the inner symbiotic bacteria generally have abundant populations and are specific microbes because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria originating from the surface have a population that is less suspected because it requires higher defense power to overcome the pathogens and predators that are around the algae.



139 Figure 3. Results of antibiotic susceptibility test against *S.aureus* and *E.coli*

Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Fernando
 B and Bruce RL, 2020). Other factors that influence the ability of inhibition are the concentration or intensity of
 antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the presence of
 organic matter and the degree of acidity (pH) (Manisha DM and Shyamapada M 2011).

144 Table 3. Results of measurement of inhibitory zone diameter of antibacterial compounds

Den etition		Diameter of zone inhibition (mm)	
Repetition	Gram positive		Gram negative

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	Symbiont bacterial	Control	Control	Symbiont bacterial	Control	Control
	(++)	(+)	(-)	(++)	(+)	(-)
1	5,5	16	0	0	13,5	0
2	7,8	17,5	0	0	14	0
Average	6,7	16,8	0	0	13,8	0

145 The area of the symptomatic supernatant inhibition zone of S.aureus was 6.7 mm. According to Mounyr Balouiri et al, 146 2016. a measured inhibition zone of less than 10 mm shows weak activity and strong activity if the the inhibition zone is 147 greater than 15 mm. Testing of antibacterial activity of the symbiont bacteria supernatant obtained was still far from the 148 results of the antibiotic activity of the chloramphenicol control. This is because the antibacterial compound of the 149 extracted symbiont bacteria was a supernatant containing secondary metabolites. However, the test results provide clear 150 evidence of antibacterial activity. Generally the chemical structure of metabolites from marine products differs from those 151 of terrestrial origin. In fact, marine bacteria are significant reservoirs of a plethora of bioactive molecules which have 152 never been found in terrestrial organisms. (Giovanna R, 2020). Seawater contains an active inhibitor agent for Gram 153 positive bacteria (Garima K et al. 2017)

154 Identification of Phenotype and Genotype of Symbiont Bacteria

Based on phenotypic identification results through cell staining and biochemical testsing, symbiont bacteria were rol shaped, non acidic, non spore forming, non motile, aerobically grown, negative catalase, and positive to carbohydrate tests. In general, the identification of selected isolates showed specific characteristics of lactic acid bacteria (*Lactobacillus* spp.), such as round colonies, milky white, Gram positive with short stem cells, without forming endospores (Davoodabadi et al. 2015).

Lactobacillus plantarum 100%

160

161 Figure 4. Sequens of 16S rDNA

162 Data for base sequence encoding gene of 16S rDNA shows that symbiont bacteria has accurate scores for species 163 levels with a similarity 100% of the sequences present in GenBank (Figure 4), The species homology of the tested isolate 164 was *Lactobacillus plantarum*.

165 In conclusion, *Turbinaria conoides* is commonly found in the gulf of Banten, Serang district, province of Banten. This 166 research showed that symbiont bacteria *Lactobacillus plantarum* are endophytic and potentially useful as an antibacterial 167 agent against common pathogens.

168

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1 Antibacterials potential of symbiont bacteria of brown algae (*Turbinaria conoides*) obtained from Indonesian waters

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Abstract. Brown seaweeds haves the potential to produce bioactive compounds. It has been shown that the bacteria associated with seaweeds are involved in the production of metabolites associated with their host. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes. In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were tested for antibacterial activity. A total of 14 isolates were isolatedfound, 6 of which came from external tissue, while 8 isolates-came from internal tissue. Through the antagonistic test, 7 isolates showed inhibitory activity against *Staphylococcuareus Staphylococcus* and *E.coli*. Phenotypic and genotypic identification showed that the species-symbiont bacteria species was *Lactobacillus plantarum*.

14 Keywords: bioassay, antagonistic, diffusion paper disc, Lactobacillus plantarum

INTRODUCTION

16 Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse, and unique 17 compounds (Bahare S et al. 2019). Many are the substances obtained from seaweed, such as alginates, carrageenan, and 18 agar, which have been used for decades in traditional medicine, pharmacology, and food (Andrea GZ et al. 2019). Other 19 compounds have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory, and antifouling activity. 20 Therefore, seaweed can provide promising bioactive that can be used in the treatment of human diseases or new 21 antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Much attention has 22 been paid to developing innovative projects for pharmaceuticals. Seaweed applications, especially in the design of new 23 antimicrobial drugs. Research for the identification of promising algal species, standardization of analytical methods, 24 isolation of compounds through integrated fractionation of bioassays, detailed chemical characterization and evaluation of 25 their safety, evaluation of synergistic effects between components, and efforts to improve yields. and lowering extraction 26 costs is needed (Marie JP et al. 2016).

27 It has been shown that the bacteribacteria associated with seaweed as epiphytes or endophytes are involved within the 28 assembly of metabolites (Alessandro B et al. 2017). It's traditionally been used for children's fever, as a fertilizer, 29 repellantrepellent, including antioxidant, anti-inflammatory, antimicrobial, and anti-cancer (Gupta et al. 2011) Turbinaria 30 conoides belongs to the family of 17the recent scientific trends target the pursuit for phytochemicals from marine algae du 31 to their numerous health-promoting effects, pathogens (Mark LW et al. 2016). Seaweeds can secrete secondary metabolite 32 with antibacterial properties (Emer S and Nissreen AG 2016). The form of symbiotic mutualism. Algae provide needed 33 sites and nutrients, while the bacteria encourage growth and protect the algal surface against symbiont bacteria isolates in 34 algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a results of infections acquired 35 from the community (Arumugama P et al. 2017) It here we evaluate the properties of the brown alga Turbinaria conoides 36 in producing bioactive compounds including the inhibition of human pathogens (Kalaivani et al. 2016). T. conoides is a 37 tropical marine algae widely distributed in coastal waters in Asia. We chose this algae following extensive trials on other 38 common macroalgae including Sargassum spp. and Euchema cottoni.

39

15

MATERIALS AND METHODS

40 Procedures

41 Sampling

42 Samples of *Turbinaria sp.* (about 1 kg wet weight) were taken from Lima island (S: -6.001051; E: 106.153804) 43 Samples were maintained in fresh seawater for laboratory analyses within 24 hours of collection.

44 Isolation of symbiont bacteria producing antibacterial compounds

45 Epibionts were extracted from 15 grams of algae by rinsin with 30 mL of sterile sea water. The rinse water was 46 incubated in 30 mL of nutrient broth medium shaken at room temperature for 24 hours. Bioactive compound was Formatted: Font: Not Bold, Italic

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extracted by crushing 15 g of algawith a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension
 was incubated with 30 mL broth nutrient medium and shaken att room temperature for 24 hours.

49 After extraction process, the refreshed samples in the 30 ml broth nutrient medium were diluted into 9 ml broth nutrient 50 sterile 10^{-1} up to 10^{-5} . Each dilutions were grown on a plate count agar medium by incubate them at 37 °C for 2 x 24 hours. 51 After incubating the petri dishes which were contain samples from each dilution, then the colonies bacteria from alga 52 would appear. The colonies bacteria producing antimicrobial compounds were characterized by a clear zone around the 53 colonies. Furthermore, the colonies with stable inhibition zones were collected by isolating them on slant agar medium, 54 with a clear code.

55 Selection of symbiont bacteria isolates antagonistically against pathogenic bacteria

To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria, a qualitative test was conducted directly by scratching round the isolates on the surface of the media that has been dispersed with test bacteria (*Eschericia coli* and *Staphylococcus aureus*)(ref). Media were incubated for 48 hours at 37 °C. Each scratching round of isolates was then marked by a unique code.

Inhibition zones were determined as those showing clear zones around the colony of symbiont bacteria isolates, for both *Escheria coli* and *Staphylococcus aureus*. Strains that showed maximum antagonistic effect against tested pathogens were identified. These were isolated and selected for further antibacterial testing by paper disc and identification of phenotype and genotype.

64 Antibacterial potential testing of symbiont bacterial isolate by paper disc diffusion

65 Testing the supernatant of symbiont bacteria for inhibitory growth of E.coli and S.aureus was performed by the agar diffusion method (Grela E et al. 2018)). The supernatant was obtained by separating the filtrate and supernatant by 66 67 centrifuge for 1 hour (25 °C and 3000 rpm). Paper discs containing supernatant 40 µL and the negative control nutrient 68 broth 40 µL were left for 1 hour to reduce the water excess, and positive control chloramphenicol 0.01 mg/mL, were 69 placed on the surface of the Mueller Hinton Agar medium containing 1 mL test bacteria and incubated for 48 hours at 37 70 C. The supernatant diffuses from the disc into the agar. If the organism is killed or inhibited by both the supernatant and 71 chloramphenicol as an antibiotic positive control, there will be no growth in the immediate area around the disc, this is 72 called the zone of inhibition. The zone sizes were compared to assess bioactivity as sensitive, resistant, or intermediate, in 73 each case the resistance zone where shows no colonies growth was measured by using a ruler to the nearest mm.

74 Identification of phenotype and genotype of symbiont bacteria

75 General bacterial identification (Phumudzo T, 2013) followed colony characteristic observations on liquid medium and 76 solid medium, observing cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and Biochemistry 77 test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase). The initial selection of isolates from mixed 78 cultures was carried out after enrichment and planting of Turbinaria conoides samples on the agar medium in pour plating. 79 Observation of medium incubated with temperature 37°C was done at incubation time reached 24 hours and 48 hours. The 80 data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from 81 Turbinaria conoides. Determination of the type of bacteria was performed based on ???. Symbiont bacteria species were 82 determined by molecular testing.

83 The DNA of the symbiont bacteria isolateds was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf 84 German) with a first pre-denaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C 85 for 30 seconds, primary attachment at 50 ° C for 30 seconds, and extension at 72 ° C for 90 seconds. After 30 cycles 86 completed, Ffollowed by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular 87 88 identification was done through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989. Modified). PCR Amplification on 16S rDNA using Primer 9 F: 5 -- AAG GAG GTG ATC 89 CAG CC-3' and Primer 1541 R: 5' - GAG TTT GAT CCT GGC TCA G - 3' (White et al., 1990, O'Donnell, 1993). The 90 91 analysis of nitrogen base sequence readings was performed with an automated DNA sequencer (ABI PRISM 3130 Genetic 92 Analyzer) (Applied Biosystems). The next sequenced raw data were trimmed and assembled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data 93 registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) 94

RESULTS AND DISCUSSION

96 The Result of Symbiont Bacteria Isolation

95

97 Samples consisted of the inside and outside of the algae, as many as 20 petri for 2 replications resulted in colonies with 98 the inhibit zone of 14 colonies, 6 of which were from epibionts, while the other 8 came from the algal tissue. The results 99 of identification of colonies grown on mixed cultures can be seen in Table 1. and identification of isolates isolated into 910 slant agar can be seen in Table 2. **Commented [A1]:** Please incert a reference paper for this procedure???

Commented [A2]: Something is missing here

101 Tabel 1. Macroscopic forms of bacterial colonies

Colonn code			Morphology of c	olonies
Colony code	Shape	Color	Edges	Elevation
TUL ² -A1-2	Round	White	Flat	Convex shiny
TUL ² -A2-2	Round	White	Flat	Convex shiny
TUL ² -A3-2	Round	White	Flat	Convex shiny
TUL ² -A4-2	Round	White	Flat	Convex shiny
TUL ² -B1-2	Round	White	Crooked	Convex shiny
TUL ² -B2-2	Round	White	Crooked	Convex shiny
TUD4-C1-2	Round	White	Flat	Convex shiny
TUD4-C2-2	Round	White	Flat	Convex shiny
TUD ² -D1-2	Round	White	Crooked	Convex shiny
TUD ² -D2-2	Round	White	Crooked	Convex shiny
TUD ² -D3-2	Round	White	Crooked	Convex shiny
TUD ² -D4-2	Round	White	Crooked	Convex shiny
TUD ⁵ -E-2	Round	White	Flat	Convex shiny
TUD ³ -F-2	Round	White	Flat	Convex shiny

102 103

Information: *The code of isolates TUL/TUD states the isolates originating from the outer/inner algae

104

** The code of isolates (2), (3), (3) states isolates obtained from the duilution *** The code of isolates (2), (3), (3) states isolates obtained from the duilution *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the 105 106 107 number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed on the plate

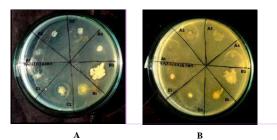
108 **** The code of number 2 identifies the isolate obtained from the second repeat

109 Table 2. Identification of the isolates on slant agar

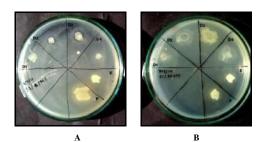
Code of isolates	Solid medium		
Code of isolates	Shape	Color	
TUL ² -A1-2	Spread	Milky white	
TUL ² -A2-2	Spread	Milky white	
TUL ² -A3-2	Spread	Milky white	
TUL ² -A4-2	Spread	Milky white	
TUL ² -B1-2	Rhizoidal	Cloudy white	
TUL ² -B2-2	Rhizoidal	Cloudy white	
TUD ⁴ -C1-2	Spread	Milky white	
TUD ⁴ -C2-2	Spread	Milky white	
TUD ² -D1-2	Rhizoidal	Cloudy white	
TUD ² -D2-2	Rhizoidal	Cloudy white	
TUD ² -D3-2	Rhizoidal	Cloudy white	
TUD ² -D4-2	Rhizoidal	Cloudy white	
TUD ⁵ -E-2	Spread	Milky white	
TUD ³ -F-2	Spread	Milky white	

110 Bacteria is isolated into a solid medium, then there is a group commonly referred to as a colony. The colony's shape is 111 different for each species and it is characteristic of a particular species (Erin RS 2012).

112 The Selection Results Symbiont Bacteria Producing Antibacterial Compounds



113 Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to S.aureus (A) and E.coli (B) **Commented [A3]:** The images are of poor quality, I would like to see in more detail these results, to determine real inhibition.



114 **Figure 2.** Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to *S.aureus* (A) and *E.coli* (B)

Based on the results of the direct challenge test, 7 bacterial isolates from 14 isolates tested showed inhibitory activity against *S.aureus* and only 2 of the 7 isolates had inhibitory activity against *E.coli*. The isolate codes that have inhibitory zones against *S.aureus* bacteria are TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2, whereas TUD4-C1-2, And TUD4-C2-2 showed inhibition zones against both pathogenic bacteria being tested but the inhibitory activity against *E.coli* was not as good as its inhibition against *S.aureus*.

Isolates with showing inhibition were re-selected by looking at the best and largest clear zone. Isolates with code 120 121 TUD4-C2-2 were isolates which had the best inhibition zone. Bacterial isolates derived from tissue showed better 122 inhibition than isolates derived from epibionts. Inhibitory zone and diameter measurement results against S.aureus and 123 E.coli can be seen in Figure 3 and Table 3. Positive controls have a broader spectrum in inhibiting both types of test bacteria with 16.8 mm inhibition against S.aureus and 13.8 mm against E.coli. Chloramphenicol with a concentration of 124 125 0.03 mg on a paper disc is highly active if its inhibition zone is more than 18 mm (Mounyr B et al., 2016), while the dose 126 of chloramphenicol (positive control) used is lower at 0.01 mg, so it can be said that bacteria Test is sensitive to positive 127 control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that a supernatant still containing medium has no effect on the activity formed. From the stability 128 129 of the measured inhibition zone, the antibacterial properties of the supernatant produced by the symbiotic bacteria act as 130 inhibitors against Gram positive bacteria and are merely bacteriostatic for Gram negative bacteria. Paper disc with a 131 supernatant applied to a Gram positive bacterial plate indicates a stable clear zone even after a 48-hour incubation period. 132 While against Gram negative bacteria, around the disc paper shows the presence of inhibitory activity but gradually become turbid before the incubation period reaches 24 hours. 133

The antibacterial compounds produced by symbiont bacteria isolates showed different inhibitory activity against both tested bacteria *S.aureus* and *E.coli*. According to Irma ESM (2011) the inner symbiotic bacteria generally have abundant populations and are specific microbes because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria originating from the surface have a population that is less suspected because it requires higher defense power to overcome the pathogens and predators that are around the algae.



139 Figure 3. Results of antibiotic susceptibility test against *S.aureus* and *E.coli*

Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Fernando
 B and Bruce RL, 2020). Other factors that influence the ability of inhibition are the concentration or intensity of
 antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the presence of
 organic matter and the degree of acidity (pH) (Manisha DM and Shyamapada M 2011).

144 Table 3. Results of measurement of inhibitory zone diameter of antibacterial compounds

Den etition		Diameter of zone inhibition (mm)	
Repetition	Gram positive		Gram negative

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	Symbiont bacterial	Control	Control	Symbiont bacterial	Control	Control
	(++)	(+)	(-)	(++)	(+)	(-)
1	5,5	16	0	0	13,5	0
2	7,8	17,5	0	0	14	0
Average	6,7	16,8	0	0	13,8	0

145 The area of the symptomatic supernatant inhibition zone of S.aureus was 6.7 mm. According to Mounyr Balouiri et al, 146 2016. a measured inhibition zone of less than 10 mm shows weak activity and strong activity if the the inhibition zone is 147 greater than 15 mm. Testing of antibacterial activity of the symbiont bacteria supernatant obtained was still far from the 148 results of the antibiotic activity of the chloramphenicol control. This is because the antibacterial compound of the 149 extracted symbiont bacteria was a supernatant containing secondary metabolites. However, the test results provide clear 150 evidence of antibacterial activity. Generally the chemical structure of metabolites from marine products differs from those 151 of terrestrial origin. In fact, marine bacteria are significant reservoirs of a plethora of bioactive molecules which have 152 never been found in terrestrial organisms. (Giovanna R, 2020). Seawater contains an active inhibitor agent for Gram 153 positive bacteria (Garima K et al. 2017)

154 Identification of Phenotype and Genotype of Symbiont Bacteria

Based on phenotypic identification results through cell staining and biochemical testsing, symbiont bacteria were rol shaped, non acidic, non spore forming, non motile, aerobically grown, negative catalase, and positive to carbohydrate tests. In general, the identification of selected isolates showed specific characteristics of lactic acid bacteria (*Lactobacillus* spp.), such as round colonies, milky white, Gram positive with short stem cells, without forming endospores (Davoodabadi et al. 2015).

Lactobacillus plantarum 100%

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161 Figure 4. Sequens of 16S rDNA

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INTRODUCTION

16 Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse, and unique 17 compounds (Bahare S et al. 2019). Many are the substances obtained from seaweed, such as alginates, carrageenan, and 18 agar, which have been used for decades in traditional medicine, pharmacology, and food (Andrea GZ-et al. 2019). Other 19 compounds have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory, and antifouling activity. 20 Therefore, seaweed can provide promising bioactive that can be used in the treatment of human diseases or new 21 22 23 24 25 26 27 28 antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Much attention has been paid to developing innovative projects for pharmaceuticals. Seaweed applications, especially are particularly used in the design of new antimicrobial drugs. Research for the identification of promising algal species, standardization of analytical methods, isolation of compounds through integrated fractionation of bioassays, detailed chemical characterization and evaluation of their safety, evaluation of synergistic effects between components, and efforts to improve yields- and lowering extraction costs is needed (Marie Pet al. 2016).

In later decades, _made strides microbiological procedures have altogether made a difference in build-up phylogenetic affiliations of seaweed-related epi bacterial communities and endophytes. Be that as it may, there's inadequately 29 30 31 32 prove that utilitarian connections for seaweed-bacterial intuitive can be built up and well caught on. Epiphytic bacterial communities are quick rapid colonizers of the ocean growth surface, some of the time versatile and able to quickly metabolize algal exudates (Singh R.P. and Reddy C.R.K, 2014). It's It has traditionally been used for children's fever, as a fertilizer, repellent, including antioxidant, anti-inflammatory, antimicrobial, and anti-cancer (Gupta et al. 2011). 33 34 35 Seaweeds can secrete secondary metabolites with antibacterial properties (Emer S- and Nissreen AG-2016). The form of symbiotic mutualism. Algae provide needed essential sites and nutrients, while the bacteria encourage growth and protec the algal surface against symbiont bacteria isolates in as algae have abundant antimicrobial activity. The existence of the 36 bacteria is suspected as a result of infections acquired from the community (Arumugama P-et al. 2017). T. conoides is 37 tropical marine alga widely distributed in coastal waters in Asia. It here we evaluate This study evaluates the properties of 38 the brown alga Turbinaria conoides in producing bioactive compounds including the inhibition of human pathogens 39 (Kalaivani et al. 2016). T. conoides is a tropical marine alga widely distributed in coastal waters in Asia. We chose this 40 alga following extensive trials on other common macroalgae including Sargassum spp. and Eucheuma cottonii.

41

MATERIALS AND METHODS

42 Procedures

43 Sampling

Samples of *Turbinaria* sp. (about 1 kg wet weight) were-was taken from Lima island (S: -6.001051; E: 106.153804
 Samples were maintained in fresh seawater for laboratory analyses analysis within 24 hours of collection.

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46 Isolation of symbiont bacteria producing antibacterial compounds

Epibionts were extracted from 15 grams of algae by rinse in with 30 mL of sterile seawater. The rinse water was
incubated in 30 mL of nutrient broth medium and shaken at room temperature for 24 hours. The bioactive compound was
extracted by crushing 15 g of alga with a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension
was incubated with 30 mL nutrient broth mutrient-medium and shaken at room temperature for 24 hours.

51 After the extraction process, the refreshed samples in the 30 ml broth nutrient medium were diluted into 9 ml broth 52 **and the extraction process, the refreshed samples in the 30 ml broth nutrient medium by incubating them at 37** $\frac{2}{3}$ C 53 for 2 x 24 hours. After incubating the petri dishes which contained samples from each dilution, then the colonies bacteria 54 from alga would appear. The colonies Colonies of bacteria producing that produce antimicrobial compounds were 55 characterized by a clear zone around the colonies. Furthermore, the colonies with stable inhibition zones were collected by 56 isolating them on a slant agar medium., with a clear code.

57 Selection of symbiont bacteria isolates antagonistically against pathogenic bacteria

To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria For this, a qualitative test was <u>conducted carried out</u> directly by scratching the isolates on the surface of the media that has been dispersed with <u>two</u> test bacteria <u>i.e.</u> (*Escherichia coli* and *Staphylococcus aureus*), (Monte-J, et al 2014)). The media <u>were-was then</u> incubated for 48 hours at 37 °C. Each scratching round of isolates was then marked by a unique code.

Inhibition zones were determined as those showing clear zones around the colony of symbiont bacteria isolates, for both *Escherichia coli* and *Staphylococcus aureus*. Strains that showed maximum antagonistic effect against tested pathogens were identified. These were isolated and selected for further antibacterial testing by paper disc and identification of phenotype and genotype. Strains showing maximum antagonistic effects were isolated and selected for antibacterial testing by paper disc method. Further the strains were identified at the phenotypic and genotypic level.

67 Antibacterial potential testing of symbiont bacterial isolate by paper disc diffusion

68 Antibacterial Testing the supernatant of symbiont bacteria for inhibitory growth of E.coli and S.aureus was 69 performed by the agar diffusion method (Grela E-et al. 2018)). The supernatant was obtained by separating the filtrate and 70 supernatant by was centrifuge for 1 hour (25 ^bC and 3000 rpm). Paper discs containing 40 µL supernatant was considered 71 as the treatment 40 µL and while 40 µL nutrient broth was used in the negative control nutrient broth 40 µL were left for 1 72 hour to reduce the water excess, and chloramphenicol (0.01 mg/mL) was used as positive control, chloramphenicol 0.01 73 mg/mL, After that the discs were placed on the surface of the Mueller Hinton Agar medium containing 1 mL test bacteria 74 and incubated for 48 hours at 37 °C. The supernatant diffuses from the disc into the agar. If the organism is killed or 75 inhibited by both the supernatant and chloramphenicol as an antibiotic positive control, there will be no growth in the 76 immediate area around the disc, this is called the zone of inhibition. The presence of a clear zone around the supernatant 77 and antibiotic discs was measured by the meter rule in mm. The zone sizes were compared to assess bioactivity as 78 sensitive, resistant, or intermediate, and the presence of a clear zone was measured by the meter rule in mm. in each case 79 the resistance zone where shows no colonies growth was measured by using a ruler to the nearest mm.

80 Identification of phenotype and genotype of symbiont bacteria

81 General bacterial identification was carried out on the basis of colony characteristic observations on liquid medium and solid medium (Phumudzo T, 2013) followed colony characteristic observations on liquid medium and solid medium, 82 83 followed by observing-cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and Biochemistry 84 biochemical test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase) as described by Phumudzo, 85 (2013). The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria 86 conoides samples on the agar medium in pour plating. Observation of medium The plates were incubated with at 37°C 87 temperature for 24 to 48 hours.37°C was done at incubation time reached 24 hours and 48 hours. The data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from Turbinaria 88 89 conoides. Determination of the type of bacteria was performed based on Phenotype and Genotype Symbiont bacteria species 90 were determined by molecular testing.

91 The DNA of the symbiont bacteria isolated was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf 92 German) with a first pre-denaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C 93 for 30 seconds, primary attachment at 50 ° C for 30 seconds, and extension at 72 ° C for 90 seconds - Followed followed 94 by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular identification was done through 95 96 partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989-Modified). PCR Amplification on 16S rDNA using Primer 9 F: 5' -- AAG GAG GTG ATC CAG CC-3' and Primer 1541 97 R: 5' - GAG TTT GAT CCT GGC TCA G - 3' (White et al., 1990, O'Donnell, 1993). The analysis of nitrogen base 98 99 sequence readings was performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied The next sequenced raw data were trimmed and assembled using the BioEdit program 100 Biosystems). (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data 101 registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) 102

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RESULTS AND DISCUSSION

104 The Result of Symbiont Bacteria Isolation

105 A total 14 colonies were isolated, of which 6 were from epibionts while the other 8 were from algal tissue. Samples

106 consisted of the inside and outside of the algae, as many as 20 petri for 2 replications resulted in colonies with the inhib

107 zone of 14 colonies, 6 of which were from epibionts, while the other 8 came from the algal tissue. The results of th 108 identification of colonies grown on mixed cultures can be seen in Table 1, and identification of isolates isolated into slar

109 agar can be seen in Table 2. The macroscopic results of colonies on mixed cultures can be seen in Table 1, and on slar

110 agar can be seen in Table 2.

111 Tabel 1. Macroscopic forms of bacterial colonies

Colorer to b			Morphology of c	olonies
Colony code	Shape	Color	Edges	Elevation
TUL ² -A1-2	Round	White	Flat	Convex shiny
TUL ² -A2-2	Round	White	Flat	Convex shiny
TUL ² -A3-2	Round	White	Flat	Convex shiny
TUL ² -A4-2	Round	White	Flat	Convex shiny
TUL ² -B1-2	Round	White	Crooked	Convex shiny
TUL ² -B2-2	Round	White	Crooked	Convex shiny
TUD4-C1-2	Round	White	Flat	Convex shiny
TUD4-C2-2	Round	White	Flat	Convex shiny
TUD ² -D1-2	Round	White	Crooked	Convex shiny
TUD ² -D2-2	Round	White	Crooked	Convex shiny
TUD ² -D3-2	Round	White	Crooked	Convex shiny
TUD ² -D4-2	Round	White	Crooked	Convex shiny
TUD ⁵ -E-2	Round	White	Flat	Convex shiny
TUD ³ -F-2	Round	White	Flat	Convex shiny

112

113 *The code of isolates TUL/TUD states the isolates originating from the outer/inner algae

114

** The code of isolates (2), (4), (5) states isolates obtained from the dilution *** The code of isolates (2), (4), (5), (3) states isolates obtained from the dilution 115 116 number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed 117 on the plate

118*** The code of number 2 identifies the isolate obtained from the second repeat

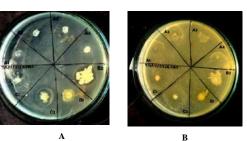
119 Table 2. Identification Macroscopic form of the isolates on slant agar

Code of isolates	Sol	id medium
Code of isolates	Shape	Color
TUL ² -A1-2	Spread	Milky white
TUL ² -A2-2	Spread	Milky white
TUL ² -A3-2	Spread	Milky white
TUL ² -A4-2	Spread	Milky white
TUL ² -B1-2	Rhizoidal	Cloudy white
TUL ² -B2-2	Rhizoidal	Cloudy white
TUD ⁴ -C1-2	Spread	Milky white
TUD ⁴ -C2-2	Spread	Milky white
TUD ² -D1-2	Rhizoidal	Cloudy white
TUD ² -D2-2	Rhizoidal	Cloudy white
TUD ² -D3-2	Rhizoidal	Cloudy white
TUD ² -D4-2	Rhizoidal	Cloudy white
TUD ⁵ -E-2	Spread	Milky white
TUD ³ -F-2	Spread	Milky white

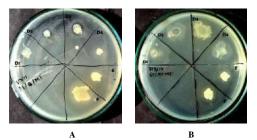
Bacteria is isolated into a solid medium, then there is a group commonly referred to as a colony. The colony's shape 120 121

different for each species and it is characteristic of a particular species (Erin RS 2012). Bacteria were isolated in a soli 122 medium and the size of the colony was different for each species and was characteristic of a particular species (Erin 2012)

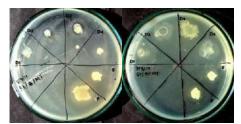
123 The selection results symbiont bacteria producing antibacterial compounds



124 Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to *S.aureus* (A) and *E.coli* (B)



- 125 **Figure 2.** Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to *S.aureus* (A) and *E.coli* (B)
- 126



127

128 Figure 2. Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to S.aureus (A) and E.coli (B)

129 Based on the results of the direct challenge test, 7 bacterial isolates from 14 isolates tested showed inhibitory activity 130 against S. aureus and only 2 of the 7 isolates had inhibitory activity against E. coli. The isolate codes that have inhibitory 131 zones against S.aureus bacteria are TUL2 B1 2, TUL2 B2 2, TUD2 D2 2, TUD2 D3 2, and TUD3 F 2, whereas TUD4 132 C1-2, And TUD4-C2-2 showed inhibition zones against both pathogenic bacteria being tested but the inhibitory activity 133 against E.coli was not as good as its inhibition against S.aureus. Based on the results of the direct challenge test, only 5 bacterial isolates i.e. TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2 showed inhibitory activity 134 135 against S.aureus whereas only 2 viz. TUD4-C1-2 and TUD4-C2-2 bacterial isolates showed inhibition zones against both pathogenic bacteria. The inhibition activity was found to be lower in E. coli than in S. aureus. 136

137 Isolates with showing inhibition were re-selected by looking at the best and largest clear zone. Isolates with code 138 TUD4-C2-2 had the best inhibition zone. Bacterial isolates derived from algal tissue showed better inhibition than isolates 139 derived from epibionts. The Inhibitory inhibitory zone and diameter measurement results against *S. aureus* and *E. coli* can 140 be seen in Figure 3 and Table 3. Positive controls have a broader spectrum in inhibiting both types of test bacteria with 16.8 mm inhibition against *S. aureus* and 13.8 mm against *E. coli*Positive controls showed 16.8 mm inhibition zone against

S. aureus and 13.8 mm inhibition zone against *E. coli*. Chloramphenicol with a concentration of 0.03 mg on a paper disc is highly active if its inhibition zone is more than 18 mm (Mounyr **B** et al., 2016), while the dose of chloramphenicol

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144 (positive control) used is was lower at less than 0.01 mg, so it can be said that bacteria Test is was found to be 145 sensitive to the positive control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of 146 activity or inhibition zone, so it can be ascertained that a supernatant still containing medium has no effect on the activit 147 formed. From the stability of the measured inhibition zone, the The antibacterial properties of the supernatant produced by 148 the symbiotic bacteria act as inhibitors against Gram-positive bacteria and are-were merely bacteriostatic for Gram 149 negative bacteria. Paper disc with a supernatant applied to a Gram-positive bacterial plate indicates a stable clear zone 150 even after a 48-hour incubation period. While against the Gram-negative bacteria, around the dise paper shows the 151 presence of inhibitory activity appeared around the disc paper, but it was gradually become turbid turbulent before the 152 incubation period reaches 24 hours.

153 The antibacterial compounds produced by symbiont bacterial isolates showed different inhibitory activity against bot 154 tested bacteria S.aureus and E.coli. According to Irma ESM et al. (2011), the inner symbiotic bacteria generally hav 155 abundant populations and are specific microbes because they directly interact with the bioactive compounds produced 156 from within the algae. While the symbiotic bacteria originating from the surface have a population that is were less 157 suspected populated, because as it requires required higher defense power to overcome the pathogens and predators that 158 are around the algae.

160 Figure 3. Results of antibiotic susceptibility test against S.aureus and E.coli

161 Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Fernando 162 B- and Bruce-RL, 2020). Other factors that influence affect the ability of inhibition potential are the concentration dr 163 intensity of antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the

164 presence of organic matter, and the degree of acidity (pH) (Manisha DM and Shyamapada M 2011).

165 Table 3. Results of measurement of inhibitory zone diameter of antibacterial compounds

	The Diameter of zone inhibition (mm)							
Repetition _	Gram	-positive		Gram-				
Repetition	Symbiont bacterial	Control	Control	Symbiont bacterial	Control	Control		
	(++)	(+)	(-)	(++)	(+)	(-)		
1	55	16	0	0	13 , .5	0		
2	7 <u>-</u> 8	17,5	0	0	14	0		
Average	6 <u>,.</u> 7	16 <u>,</u> 8	0	0	13 <u>,</u> 8	0		

The area of the symptomatic supernatant inhibition zone of S.aureus was 6.7 mm. According to Mounyr Balouiri et a 166 (2016)- a measured-less than 10 mm inhibition zone of less than 10 mm shows showed weak activity and strong activity 167 168 the inhibition zone is greater than 15 mm it indicates strong activity. Testing of antibacterial activity of the symbion 169 bacteria supernatant obtained was still far from the results of the antibiotic activity of the chloramphenicol control. This is 170 because the antibacterial compound of the extracted symbiont bacteria was a the supernatant containing secondary 171 metabolites. However, the test results provide clear evidence of antibacterial activity. Generally, the chemical structure of 172 metabolites from marine products differs from those of terrestrial origin. Marine bacteria are significant reservoirs of a 173 plethora of bioactive molecules that have never been found in terrestrial organisms- (Giovanna R, 2020). Seawate 174 contains an active inhibitor agent for Gram-positive bacteria (Garima K et al. 2017)

175 Identification of Phenotype and Genotype of Symbiont Bacteria

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177 Based on the phenotypic observation comes about of phenotypic recognizable proof through cell recoloring and 178 biochemical tests, the symbiont microscopic organisms were rod-shaped, non-acidic, non-spore-forming, non

motile, developing grow vigorously, catalase-negative, and a positive test for carbohydrates. In common, 179 180 the distinguishing proof of chosen segregates appeared particular characteristics of lactic corrosive microscopic Commented [K8]: Instead of Garima you should write

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181	organisms (Lactobacillus	spp.),	Such	as circular, smooth white,	Gram-positive	colonies	with brief stem	cells,
182	without shaping endospore	s (Davo	odabadi	et al. 2015).				

183 The Genotypic result through molecular identification is carried outwas done through partial genetic analysis of 16S rDNA. PCR amplification results from the 16S region of bacterial ribosome DNA_Nitrogen base sequences sorted from 184 185 symbiont bacterial isolates can be seen in figure 4. The sequencing information was sequenced in impact with under the 186 influence of genomic information enlisted within the DDBJ / Japanese DNA Information Bank with 100% 187 strains grouping comes about. The characters of the antibacterial strains were indistinguishable from those of Lactobacillus 188 plantarum. Greatest Highest 100% personality, greatest score 2660, add up to score 2660, 100% inquiry scope, E esteem 0, 189 was recorded to for the taxon of adjacent microbes. The classification of bacterial confines is as takes after: Microscopic 190 organisms; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus plantarum. 191

192 Sequens of 16S rDNA

193 194 TTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAG 195 ATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCG 196 CGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACA 197 TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAG 198 CAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCA 199 GGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG 200 TCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTG 201 CATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA 202 203 AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAG ATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCAT 204 TAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC 205 ATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCC 206 TTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG 207 CAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA 208 209 AGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGT 210AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACA 211 CCCAAAGTC

Figure 4. The arrangement of nitrogen bases sequenced from symbiont bacteria, A = adenine, T = thiamine, G = guanine, C = cytosine.

Data for base sequence encoding gene of 16S rDNA shows that symbiont bacteria have accurate scores for species levels with a similarity 100% of the sequences present in GenBank. The species homology of the tested isolate was *Lactobacillus plantarum. Lactobacillus plantarum* strains separated from dairy items appeared solid antimicrobial action against the pointers strains of *Staphylococcus aureus*, *Salmonella spp*, and *Escherichia coli* (Hu C.H., et al 2019). The separation isolation of *L. plantarum* from Tibetan yaks was able to restrain the development of *E. coli and S. aureus* (Wang L., et al 2018). Few-Some Lactobacillus _lactobacillus _strains appeared showed antibacterial movement against Enterobacteriaceae which that were safe to for carbapenems (CRE). This impact effect may have potential applications through the utilize of the Lactobacillus strain as a starter culture in aged nourishments or as a nourishment additive to control or avoid CRE contamination (Chen C-C., et al 2019).

In conclusion, *Turbinaria conoides* is was commonly found in the gulf of Banten, Serang district, province of Banten.
 This research showed revealed that symbiont bacteria *Lactobacillus plantarum* are was endophytic and potentially useful as an antibacterial agent against common pathogens.

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Antibacterial potential of symbiont bacteria of brown algae (*Turbinaria conoides*) obtained from Indonesian waters

Abstract. Brown seaweeds have the potential to produce bioactive compounds. It has been shown that the bacteria Bacteria associated with seaweeds are involved in the production of metabolites associated with their host. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes. In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were tested for antibacterial activity. A total of of 14 isolates were found bacteria were isolated—, 6-of which eame 6 were isolated from external tissue, while 8 eame-from internal tissue. Through the Results of an antagonistic test revealed that τ_{-} 7 isolates showed inhibitory activity against *Staphylococcus aureus* and only 1 isolate showed the inhibition against both *S.aureus* and *E.coli*. Phenotypic and genotypic identification analysis showed that the symbiont bacteria species was *Lactobacillus plantarum*.

4 Keywords: bioassay Bioassay, brown seaweed, antagonistic, diffusion paper disc, Lactobacillus plantarum

INTRODUCTION

Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse, and unique 16 17 compounds (Bahare S et al. 2019). Many are the substances are obtained from seaweed, such as alginates, carrageenar 18 and agar, which have been used for decades in traditional medicine, pharmacology, and food (Andrea GZ et al. 2019). 19 Other compounds have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory, and antifouling activity. 20 Therefore, seaweed can provide promising bioactive that can be used in the treatment of human diseases or new 21 22 23 24 25 26 27 28 antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Much attention has been paid to developing innovative projects for pharmaceuticals. Seaweed applications, especially are particularly used in the design of new antimicrobial drugs. Research for the identification of promising algal species, standardization of analytical methods, isolation of compounds through integrated fractionation of bioassays, detailed chemical characterization and evaluation of their safety, evaluation of synergistic effects between components, and efforts to improve yields- and lowering extraction costs is needed (Marie Pet al. 2016).

In later decades, _made strides microbiological procedures have altogether made a difference in build-up phylogenetic affiliations of seaweed-related epi bacterial communities and endophytes. Be that as it may, there's inadequately 29 30 31 32 prove that utilitarian connections for seaweed-bacterial intuitive can be built up and well caught on. Epiphytic bacterial communities are quick rapid colonizers of the ocean growth surface, some of the time versatile and able to quickly metabolize algal exudates (Singh R.P. and Reddy C.R.K, 2014). It's It has traditionally been used for children's fever, as a fertilizer, repellent, including antioxidant, anti-inflammatory, antimicrobial, and anti-cancer (Gupta et al. 2011). 33 34 35 Seaweeds can secrete secondary metabolites with antibacterial properties (Emer S-and Nissreen AG-2016). The formsymbiotic mutualism occurs as - Aalgae provide needed essential sites and nutrients, while the bacteria encourage growt and protect the algal surface against symbiont bacteria isolates in as algae have abundant antimicrobial activity. The 36 existence of the bacteria is suspected as a result of infections acquired from the community (Arumugama P-et al. 2017). 37 conoides is a tropical marine alga widely distributed in coastal waters in Asia. It here we evaluate This study evaluates the 38 properties of the brown alga Turbinaria conoides in producing bioactive compounds including the inhibition of human 39 pathogens (Kalaivani et al. 2016). T. conoides is a tropical marine alga widely distributed in coastal waters in Asia. We 40 chose this alga following extensive trials on other common macroalgae including Sargassum spp. and Eucheuma cottonii.

MATERIALS AND METHODS

42 Procedures

43 Sampling

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Samples of *Turbinaria* sp. (about 1 kg wet weight) were were was taken from Lima island (S: -6.001051; E
 106.153804) Samples were maintained in fresh seawater for laboratory analyses analysis within 24 hours of collection.

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46 Isolation of symbiont bacteria producing antibacterial compounds

Epibionts were extracted from 15 grams of algae by rinse in with 30 mL of sterile seawater. The rinse water was incubated in 30 mL of nutrient broth medium and shaken at room temperature for 24 hours. The bioactive compound was extracted by crushing 15 g of alga with a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension was incubated with put into a 30 ml nutrient broth mutrient medium and shaken at room temperature for 24 hours.

After the extraction process, <u>1 ml of</u> the refreshed samples <u>from in</u>-the 30 ml broth nutrient <u>was measured out and</u> homogenized in the sterile test tube containing 9 ml of medium were diluted in stages in sterile theto 9 ml putrient broth, to produce a 10⁻¹ dilution. This was done until 10⁻⁸ dilution is produced. <u>-for each dilute</u> <u>hutrient broth sterile 10⁻⁴ up to 10⁻⁸</u>. Each dilution was grown on a plate count agar medium by incubating them at 37 <u>°</u>^cC for 2 x 24 hours. <u>After incubating</u> the petri dishes which contained samples from each dilution, then the colonies bacteria from alga would appear. The colonies <u>Colonies of</u> bacteria <u>producing that produce</u> antimicrobial compounds were characterized by a clear zone-around

the colonies of bacteria producing that produce and p

59 Selection of symbiont bacteria isolates antagonistically against pathogenic bacteria

To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria For this, a qualitative test was conducted carried out directly by scratching the isolates on the surface of the media that has been dispersed with two test bacteria i.e. (Escherichia coli and Staphylococcus aureus), (Monte-J, et al 2014)). The media were-was then incubated for 48 hours at 37 °C. Each scratching round of isolates was then marked by a unique code.

Inhibition zones were determined as those showing clear zones around the colony of symbiont bacteria isolates, for both *Escherichia coli* and *Staphylococcus aureus*. Strains that showed maximum antagonistic effect against tested pathogens were identified. These were isolated and selected for further antibacterial testing by paper disc and identification of phenotype and genotype. Strains showing maximum antagonistic effects were isolated and selected for- antibacterial testing by the paper disc diffusion method. Further, the strains were identified at the phenotypic and genotypic levels.

69 Antibacterial potential testing of symbiont bacterial isolate by paper disc diffusion

70 Antibacterial Testing the supernatant of symbiont bacteria for inhibitory growth of E.coli and S.aureus was 71 performed by the ager-paper disc diffusion method (Grela E-et al. 2018)-). The supernatant was obtained by separating the 72 filtrate and the -supernatant by was centrifuged for 1 hour (25 ²°C and 3000 rpm). Paper discs containing 40 µL supernatant 73 was considered as the treatment 40 µL and while 40 µL nutrient broth was used in-the negative control nutrient broth 40 74 HL were left for 1 hour to reduce the water excess, and chloramphenicol (0.01 mg/mL) was used as a positive control. 75 chloramphenicol 0.01 mg/mL, After that, the discs were placed on the surface of the Mueller Hinton Agar medium 76 containing 1 mL test bacteria and incubated for 48 hours at 37 °C. The supernatant diffuses from the disc into the agar.-If 77 the organism is killed or inhibited by both the supernatant and chloramphenicol as an antibiotic positive control, there will 78 be no growth in the immediate area around the dise, this is called the zone of inhibition. The presence of a clear zone 79 around the supernatant and antibiotic discs was measured by the meter rule in mm. The zone sizes were compared to 80 assess bioactivity as sensitive, resistant, or intermediate, and the presence of a clear zone was measured by the meter rule 81 in mm. in each case the resistance zone where shows no colonies growth was measured by using a ruler to the nearest mm.

82 Identification of phenotype and genotype of symbiont bacteria

83 General bacterial identification was carried out based on on the basis of colony characteristic observations on liquid 84 medium and solid medium (Phumudzo T, 2013) followed colony characteristic observations on liquid medium and solid medium, followed by observing cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and 85 86 Biochemistry biochemical test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase) as described by 87 Phumudzo, (2013). The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria conoides samples on the agar medium in pour plating. Observation of mediumThe plates were incubated with 88 89 at 37°C temperature for 24 to 48 hours. 37°C was done at incubation time reached 24 hours and 48 hours. The data obtained 90 from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from Turbinaria 91 conoides. Determination of the type of bacteria was performed based on Phenotype and Genotype Symbiont bacteria species 92 were determined by molecular testing.

The DNA of the symbiont bacteria isolated was amplified using primers 9F and 1541R. The DNA bands used were 93 94 relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf 95 German) with a first pre-denaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C 96 for 30 seconds, primary attachment at 50 ° C for 30 seconds, and extension at 72 ° C for 90 seconds, - Followed followed 97 by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular identification was done through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989-98 99 Modified). PCR Amplification on 16S rDNA using Primer 9 F: 5' -- AAG GAG GTG ATC CAG CC-3' and Primer 1541 100 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 performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied 101 102 The next sequenced raw data were trimmed and assembled using the BioEdit program Biosystems).

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103 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data 104 registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/)

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RESULTS AND DISCUSSION

106 The Result of Symbiont Bacteria Isolation

107 A total of 14 colonies were isolated, of which 6 were from epibionts while the other 8 were from algal tissue. Samples 108 consisted of the inside and outside of the algae, as many as 20 petri for 2 replications resulted in colonies with the inhibit

109 zone of 14 colonies, 6 of which were from epibionts, while the other 8 came from the algal tissue. The results of the

identification of colonies grown on mixed cultures can be seen in Table 1, and identification of isolates isolated into slar 110 111 agar can be seen in Table 2. The macroscopic results of colonies on mixed cultures can be seen in Table 1, and on slar

112 agar can be seen in Table 2.

113 Tabel 1. Macroscopic forms of bacterial colonies

Colonn orde		Morphology of colonies				
Colony code	Shape	Color	Edges	Elevation		
TUL ² -A1-2	Round	White	Flat	Convex shiny		
TUL ² -A2-2	Round	White	Flat	Convex shiny		
TUL ² -A3-2	Round	White	Flat	Convex shiny		
TUL ² -A4-2	Round	White	Flat	Convex shiny		
TUL ² -B1-2	Round	White	Crooked	Convex shiny		
TUL ² -B2-2	Round	White	Crooked	Convex shiny		
TUD4-C1-2	Round	White	Flat	Convex shiny		
TUD4-C2-2	Round	White	Flat	Convex shiny		
TUD ² -D1-2	Round	White	Crooked	Convex shiny		
TUD ² -D2-2	Round	White	Crooked	Convex shiny		
TUD ² -D3-2	Round	White	Crooked	Convex shiny		
TUD ² -D4-2	Round	White	Crooked	Convex shiny		
TUD ⁵ -E-2	Round	White	Flat	Convex shiny		
TUD ³ -F-2	Round	White	Flat	Convex shiny		

114

*The code of isolates TUL/TUD states the isolates originating from the outer/inner algae 115

116 ** The code of isolates (²), (⁴), (⁵), (³) states isolates obtained from the dilution

117 *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the 118 number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed 119 on the plate

120 **** The code of number 2 identifies the isolate obtained from the second repeat

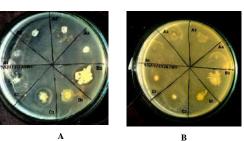
121 Table 2. Identification Macroscopic form of the isolates on slant agar

Code of isolates	Sol	id medium
Code of isolates	Shape	Color
TUL ² -A1-2	Spread	Milky white
TUL ² -A2-2	Spread	Milky white
TUL ² -A3-2	Spread	Milky white
TUL ² -A4-2	Spread	Milky white
TUL ² -B1-2	Rhizoidal	Cloudy white
TUL ² -B2-2	Rhizoidal	Cloudy white
TUD ⁴ -C1-2	Spread	Milky white
TUD ⁴ -C2-2	Spread	Milky white
TUD ² -D1-2	Rhizoidal	Cloudy white
TUD ² -D2-2	Rhizoidal	Cloudy white
TUD ² -D3-2	Rhizoidal	Cloudy white
TUD ² -D4-2	Rhizoidal	Cloudy white
TUD ⁵ -E-2	Spread	Milky white
TUD ³ -F-2	Spread	Milky white

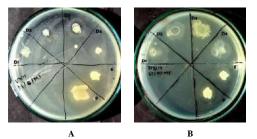
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Bacteria is isolated into a solid medium, then there is a group commonly referred to as a colony. The colony's shape 123 different for each species and it is characteristic of a particular species (Erin RS 2012). Bacteria were isolated in a sol 124 medium and the size of the colony was different for each species and was characteristic of a particular species (Erin 2012)

125 The selection results symbiont bacteria producing antibacterial compounds



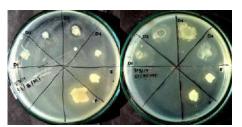
126 Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to *S. aureus* (A) and *E. coli* (B)



127 **Figure 2.** Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to *S.aureus* (A) and *E.coli* (B)

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130 Figure 2. Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to S.aureus (A) and E.coli (B)

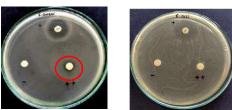
131 Based on the results of the direct challenge test, 7 bacterial isolates from 14 isolates tested showed inhibitory activity 132 against S. aureus and only 2 of the 7 isolates had inhibitory activity against E. coli. The isolate codes that have inhibitory 133 zones against S.aureus bacteria are TUL2 B1 2, TUL2 B2 2, TUD2 D2 2, TUD2 D3 2, and TUD3 F 2, whereas TUD4 134 C1-2, And TUD4-C2-2 showed inhibition zones against both pathogenic bacteria being tested but the inhibitory activity 135 against E.coli was not as good as its inhibition against S.aureus. Based on the results of the direct challenge test, only 5 bacterial isolates i.e. TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2 showed inhibitory activity 136 against S.aureus whereas only 2 viz. TUD4-C1-2 and TUD4-C2-2 bacterial isolates showed inhibition zones against both 137 pathogenic bacteria. The inhibition activity was found to be lower in E. coli than in S. aureus. 138

139 Isolates with showing inhibition were re-selected by looking at the best and largest clear zone. Isolates with code 140 TUD4-C2-2 had the best inhibition zone. Bacterial isolates derived from algal tissue showed better inhibition than isolates 141 derived from epibionts. The Inhibitory inhibitory zone and diameter measurement results against *S. aureus* and *E. coli* can 142 be seen in Figure 3 and Table 3. Positive controls have a broader spectrum in inhibiting both types of test bacteria with 143 16.8 mm inhibition against *S. aureus* and 13.8 mm against *E. coli*Positive controls showed 16.8 mm inhibition zone against

S. aureus and 13.8 mm inhibition zone against *E. coli*. Chloramphenicol with a concentration of 0.03 mg on a paper disc is highly active if its inhibition zone is more than 18 mm (Mounyr B- et al., 2016), while the dose of chloramphenicol Formatted: Font: Italic

146 (positive control) used is was lower at less than 0.01 mg, so it can be said that bacteria Test is was found to be 147 sensitive to the positive control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of 148 activity or inhibition zone, so it can be ascertained that a supernatant still containing medium has no effect ondoes n 149 affect the activity formed. -From the stability of the measured inhibition zone, the The antibacterial properties of the 150 supernatant produced by the symbiotic bacteria act as inhibitors against Gram-positive bacteria and are-were merel 151 bacteriostatic for Gram-negative bacteria. As gram-positive symbiotic bacteria widely knows contain bacterioci 152 (Mezaini A et al, 2009 and Li D. Et al, 2015) bacteriocins from Gram-positive bacteria are generally not effective agair 153 Gram-negative bacteria (Smaoui et al, 2010). Paper disc with a supernatant applied to a Gram-positive bacterial plat 154 indicates a stable clear zone even after a 48-hour incubation period. While against the Gram-negative bacteria, around the 155 disc paper shows the presence of inhibitory activity <u>appeared around the disc paper</u>, but <u>it was</u> gradually become turbi 156 turbulent before the incubation period reaches 24 hours.

The antibacterial compounds produced by symbiont bacterial isolates showed different inhibitory activity against both tested bacteria *S.aureus* and *E.coli*. According to Irma ESM et al. (2011), the inner symbiotic bacteria generally have abundant populations and are specific microbes because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria originating from the surface have a population that is were less suspected populated, because as it requires required higher defense power to overcome the pathogens and predators that are around the algae.



164 Figure 3. Results of antibiotic susceptibility test against S.aureus and E.coli

165 Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Fernando

B- and Bruce RL, 2020). Other factors that influence affect the ability of inhibition potential are the concentration dr

167 intensity of antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the

168 presence of organic matter, and the degree of acidity (pH) (Manisha DM and Shyamapada M 2011).

169 Table 3. Results of measurement of inhibitory zone diameter of antibacterial compounds

		The	Diameter of zor	ne inhibition (mm)		
Repetition -	Gram	-positive		Gram-negative		
Repetition	Symbiont bacterial	Control	Control	Symbiont bacterial	Control	Control
	(++)	(+)	(-)	(++)	(+)	(-)
1	5 <u>, 5</u>	16	0	0	13,5	0
2	7 <u>.</u> 8	17 <u>, 5</u>	0	0	14	0
Average	6 <u>.</u> 7	16 <u>,.</u> 8	0	0	13 <u>,</u> 8	0

170 The area of the symptomatic supernatant inhibition zone of S.aureus was 6.7 mm. According to Mounyr Balouiri et a 171 (2016), - a measured less than 10 mm inhibition zone of less than 10 mm shows showed weak activity and strong activity the inhibition zone is greater than 15 mm_it indicates strong activity. Testing of antibacterial activity of the symbiont 172 173 bacteria supernatant obtained was still far from the results of the antibiotic activity of the chloramphenicol control. This is 174 because_of_the antibacterial compound of the extracted symbiont bacteria was a_tthe supernatant containing secondary 175 metabolites. However, the test results provide clear evidence of antibacterial activity. Generally, the chemical structure of 176 metabolites from marine products differs from-the those of terrestrial origin. Marine bacteria are significant reservoirs of 177 plethora of bioactive molecules that have never been found in terrestrial organisms- (Giovanna R, 2020). Seawate 178 contains an active inhibitor agent for Gram-positive bacteria (Garima KKapoor et al. 2017)

179 Identification of Phenotype and Genotype of Symbiont Bacteria

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184 motile, developing and grow vigorouslaerobically, negative catalase, and positive carbohydrate testy., catalase negative, 185 and a positive test for carbohydra tes. In general, the selected isolate showed special characteristics possessed by lactic 186 acid bacteria common, the distinguishing proof of chosen segregates appeared particular characteristics lactic corrosive microscopic organisms (Lactobacillus spp.), s-Such_as _circular, _smooth _white, Gram-positive colonies 187 188 with_brief _stem cells, _without_shaping endospores (Davoodabadi et al. 2015). 189 190 The Genotypic result through molecular identification is carried outwas done through partial genetic analysis of 16S 191 rDNA. PCR amplification results from the 16S region of bacterial ribosome DNA_Nitrogen base sequences sorted from 192 symbiont bacterial isolates can be seen in figure 4. The sequencing information was sequenced in impact with under the 193 influence of genomic information enlisted within the DDBJ / Japanese DNA Information Bank with 100% 194 strains grouping comes about. The characters of the antibacterial strains were indistinguishable from those of Lactobacillus 195 plantarum. Greatest. The hHighest 100% personality, greatest score 2660, add up to score 2660, 100% inquiry scope, 196 E esteem 0, was recorded to for the taxon of adjacent microbes. The classification of -of-the bacterial isolate is Bacteria; 197 Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus plantarum. bacterial confines is as takes after. Microscopic organisms; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; 198 199 Lactobacillus; Lactobacillus plantarum. 200 201 Sequens of 16S rDNA 202 203 TTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGGATAACACCTGGAAACAG 204 205 ATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCG CGGCGTATTAGCTAGATGGTGGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACA 206 TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAG 207 CAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCA 208 209 $GGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG\\TCCGGATTTATTGGGCGTAAAGCGAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTG$ 210 CATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA 211 212 AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAG ATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCAT TAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC 213 214 ATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCC 215 TTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG 216 CAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGGA 217 218 AGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGT 219 220 CCCAAAGTC

221 Figure 4. The arrangement of nitrogen bases sequenced from symbiont bacteria, A = adenine, T = thiamine, G = guanine, C = cytosine. 222

223 224 225 226 227 228 Data for base sequence encoding gene of 16S rDNA shows that symbiont bacteria have accurate scores for species levels with a similarity of 100% of the sequences present in GenBank. The species homology of the tested isolate was Lactobacillus plantarum. Lactobacillus plantarum strains separated from dairy items appeared solid antimicrobial action against the pointers strains of Staphylococcus aureus, Salmonella spp, and Escherichia coli (Hu C.H., et al 2019). The separation-isolation of L. plantarum from Tibetan yaks was able to restrain the development of E. coli and S. aureus (Wang L, et al 2018). Few Some LLactobacillus lactobacillus _strains appeared showed antibacterial movement against 229 Enterobacteriaceae which that were safe to for carbapenems (CRE). This impact effect may have potential applications 230 through the utilize of the Lactobacillus strain as a starter culture in aged nourishments or as a nourishment additive to 231 control or avoid CRE contamination (Chen C-C, et al 2019).

232 In conclusion, Turbinaria conoides was is was commonly found in the gulf of Banten, Serang district, province of 233 Banten. This research showed revealed that symbiont bacteria Lactobacillus plantarum are was endophytic and potentially 234 useful as an antibacterial agent against common pathogens.

ACKNOWLEDGEMENTS

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Antibacterial potential of symbiont bacteria of brown algae (*Turbinaria conoides*) obtained from Indonesian waters

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Abstract. Dharmayanti N, Anti A, Siregar RR, Sipahutar Y, Permadi A, Siregar AN, Salampessy RB, Sujuliyanti, Nurbani SZ, Purnamasari HB. 2021. Title. Biodiversitas 22: 373-377. Brown seaweeds have the potential to produce bioactive compounds. Bacteria associated with seaweeds are involved in the production of metabolites. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes. In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were tested for antibacterial activity. A total of 14 bacteria were isolated, of which 6 were isolated from external tissue, while 8 from internal tissue. Results of an antagonistic test revealed that 7 isolates showed inhibitory activity against *Staphylococcus aureus* and only 1 isolate showed the inhibition against both *S. aureus* and *Escherichia coli*. Phenotypic and genotypic analysis showed that the symbiont bacteria was *Lactobacillus plantarum*.

Keywords: Bioassay, brown seaweed, antagonistic, diffusion paper disc, Lactobacillus plantarum

INTRODUCTION

Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse, and unique compounds (Bahare et al. 2019). Many substances are obtained from seaweed, such as alginates, carrageenan, and agar, which have been used for decades in traditional medicine, pharmacology, and food (Andrea et al. 2019). Other compounds have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory, and antifouling activity. Therefore, seaweed can provide promising bioactive that can be used in the treatment of human diseases or new antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Seaweed applications are particularly used in the design of new antimicrobial drugs. Research for the identification of promising algal species, standardization of analytical methods, isolation of compounds through integrated fractionation of bioassays, detailed chemical characterization and evaluation of their safety, evaluation of synergistic effects between components, and efforts to improve yields and lowering extraction costs is needed (Marie et al. 2016).

In later decades, strides microbiological procedures have altogether made a difference in build-up phylogenetic affiliations of seaweed-related epi-bacterial communities and endophytes. Be that as it may, there is inadequately prove that utilitarian connections for seaweed-bacterial intuitive can be built up and well caught on. Epiphytic bacterial communities are rapid colonizers of the ocean growth surface, some of the time versatile and able to quickly metabolize algal exudates (Singh and Reddy 2014). It has traditionally been used for children's fever, as a fertilizer, repellent, including antioxidant, anti-inflammatory, antimicrobial, and anti-cancer (Gupta and Abu-Ghannam 2011). Seaweeds can secrete secondary metabolites with antibacterial properties (Shannon and Abu-Ghannam 2016). The symbiotic mutualism occurs as algae provide essential sites and nutrients, while the bacteria encourage growth and protect the algal surface against symbiont bacteria isolates as algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of infections acquired from the community (Arumugama et al. 2017). *Turbinaria conoides* is a tropical marine alga widely distributed in coastal waters in Asia.

This study evaluates the properties of the brown alga *Turbinaria conoides* in producing bioactive compounds including the inhibition of human pathogens.

MATERIALS AND METHODS

Sampling

Samples of *Turbinaria conoides* (about 1 kg wet weight) were taken from Lima island, Serang City, Banten, Indonesia (S: -6.001051; E: 106.153804). Samples were maintained in fresh seawater for laboratory analysis within 24 hours of collection.

Isolation of symbiont bacteria producing antibacterial compounds

Bacteria were isolated in a solid medium and the size of the colony was different for each species and was characteristic of a particular species (Sanders 2012). Epibionts were extracted from 15 grams of algae by rinse in with 30 mL of sterile seawater. The rinse water was incubated in 30 mL of nutrient broth medium and shaken at room temperature for 24 hours. The bioactive compound was extracted by crushing 15 g of alga with a mortar and pestle with the addition of 15 mL of sterile seawater. The suspension was insert into a 30 mL nutrient broth medium and shaken at room temperature for 24 hours.

After the extraction process, 1 mL of refresh samples were diluted in a 9 mL of sterile nutrient broth to make 10^{-1} dilution. This process was continued to achieve 10^{-5} dilutionEach dilution was grown on a plate count agar medium by incubating them at 37° C for 2 x 24 hours. Colonies of bacteria that produce antimicrobial compounds were characterized by a clear zone. Furthermore, the colonies with stable inhibition zones were collected by isolating them on a slant agar medium.

Selection of symbiont bacteria isolates antagonistically against pathogenic bacteria

For this, a qualitative test was carried out directly by scratching the isolates on the surface of the media that has been dispersed with two test bacteria, i.e. *Escherichia coli* and *Staphylococcus aureus* (Monte et al. 2014)). The media was then incubated for 48 hours at 37°C. Each scratching round of isolates was then marked by a unique code. Inhibition zones were showing clear zones around the colony of symbiont bacteria isolates for both *E. coli* and *S. aureus*. Strains that showed maximum antagonistic effect against tested pathogens were identified. Strains showing maximum antagonistic effects were isolated and selected for antibacterial testing by the paper disc diffusion method. Further, the strains were identified at the phenotypic and genotypic levels.

Antibacterial potential testing of symbiont bacterial isolate by paper disc diffusion

Antibacterial testing of symbiont bacteria for inhibitory growth of E. coli and S. aureus was performed by the paper disc diffusion method (Grela et al. 2018). The supernatant was obtained by separating the filtrate and the supernatant was centrifuged for 1 hour (25°C and 3000 rpm). Paper discs containing 40 µL supernatant was considered as the treatment while 40 uL nutrient broth was used in negative control and chloramphenicol (0.01 mg/mL) was used as a positive control. After that, the discs were placed on the surface of the Mueller Hinton Agar medium containing 1 mL test bacteria and incubated for 48 hours at 37°C. The supernatant diffuses from the disc into the agar. The presence of a clear zone around the supernatant and antibiotic discs was measured by the meter rule in mm. The zone sizes were compared to assess bioactivity as sensitive, resistant, or intermediate, and the presence of a clear zone was measured by the meter rule in mm.

Identification of symbiont bacteria phenotype and genotype

General bacterial identification based on colony characteristic observations on liquid medium and solid medium followed by cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and biochemical test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase) as described by Phumudzo et al. (2013). The initial selection of isolates from mixed cultures was carried out after enrichment and planting of T. conoides samples on the agar medium. The plates were incubated at 37°C temperature for 24 to 48 hours. The data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from T. conoides. The DNA of the symbiont bacteria isolated was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf German) with a first pre-denaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, primary attachment at 50°C for 30 seconds, and extension at 72°C for 90 seconds, followed by the elongation phase at 72°C for 5 min and cooling at 4°C for 20 min. Molecular identification was done through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al. 1989 modified). 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 performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The next sequenced raw data were trimmed and assembled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data registered in DDBJ/DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/).

RESULTS AND DISCUSSION

The result of symbiont bacteria isolation

A total of 14 colonies were isolated, of which 6 were from epibionts while the other 8 were from algal tissue. The macroscopic results of colonies on mixed cultures can be seen in Table 1, and on slant agar can be seen in Table 2.

The selection results symbiont bacteria producing antibacterial compounds

Based on the results of the direct challenge test, only 5 bacterial isolates i.e. TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2 showed inhibitory activity against *S.aureus* whereas only 2 viz. TUD4-C1-2 and TUD4-C2-2 bacterial isolates showed inhibition zones against both pathogenic bacteria. The inhibition activity was found to be lower in *E. coli* than in *S. aureus* (Figures 1 and 2).

Isolates with code TUD4-C2-2 had the best inhibition zone. Bacterial isolates derived from algal tissue showed better inhibition than isolates derived from epibionts. The inhibitory zone and diameter measurement results against *S. aureus* and *E. coli* can be seen in Figure 3 and Table 3.

Positive controls showed 16.8 mm inhibition zone against *S. aureus* and 13 8 mm inhibition zone against *E. coli*. Chloramphenicol with a concentration of 0.03 mg on a

paper disc is highly active if its inhibition zone is more than 18 mm (Mounyr et al. 2016), while the dose of chloramphenicol (positive control) used was less than 0.01 mg, so it can be said that bacteria test was found to be sensitive to the positive control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that supernatant does not affect the activity formed.

Tabel 1. Macroscopic forms of bacterial colonies.

Colony		Morph	ology of col	onies
code	Shape	Color	Edges	Elevation
TUL ² -A1-2	Round	White	Flat	Convex shiny
TUL ² -A2-2	Round	White	Flat	Convex shiny
TUL ² -A3-2	Round	White	Flat	Convex shiny
TUL ² -A4-2	Round	White	Flat	Convex shiny
TUL ² -B1-2	Round	White	Crooked	Convex shiny
TUL ² -B2-2	Round	White	Crooked	Convex shiny
TUD ⁴ -C1-2	Round	White	Flat	Convex shiny
TUD ⁴ -C2-2	Round	White	Flat	Convex shiny
TUD ² -D1-2	Round	White	Crooked	Convex shiny
TUD ² -D2-2	Round	White	Crooked	Convex shiny
TUD ² -D3-2	Round	White	Crooked	Convex shiny
TUD ² -D4-2	Round	White	Crooked	Convex shiny
TUD ⁵ -E-2	Round	White	Flat	Convex shiny
TUD ³ -F-2	Round	White	Flat	Convex shiny

Note: *The code of isolates TUL/TUD states the isolates originating from the outer/inner algae. ** The code of isolates $(^2)$, $(^4)$, $(^5)$, $(^3)$ states isolates obtained from the dilution. *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed on the plate. **** The code of number 2 identifies the isolate obtained from the second repeat

Table 2. Macroscopic form of the isolates on slant agar

Code of isolates	Solid medium				
Code of isolates	Shape	Color			
TUL ² -A1-2	Spread	Milky white			
TUL ² -A2-2	Spread	Milky white			
TUL ² -A3-2	Spread	Milky white			
TUL ² -A4-2	Spread	Milky white			
TUL ² -B1-2	Rhizoidal	Cloudy white			
TUL ² -B2-2	Rhizoidal	Cloudy white			
TUD ⁴ -C1-2	Spread	Milky white			
TUD ⁴ -C2-2	Spread	Milky white			
TUD ² -D1-2	Rhizoidal	Cloudy white			
TUD ² -D2-2	Rhizoidal	Cloudy white			
TUD ² -D3-2	Rhizoidal	Cloudy white			
TUD ² -D4-2	Rhizoidal	Cloudy white			
TUD ⁵ -E-2	Spread	Milky white			
TUD ³ -F-2	Spread	Milky white			

Table 3. Results of inhibitory zone diameter

	The diameter of zone inhibition (mm)						
Donotition	Gram-	positive		Gram-negative			
Repetition -	Symbiont bacterial	Control	Control	Symbiont bacterial	Control	Control	
	(++)	(+)	(-)	(++)	(+)	(-)	
1	5.5	16	0	0	13.5	0	
2	7.8	17.5	0	0	14	0	
Average	6.7	16.8	0	0	13.8	0	

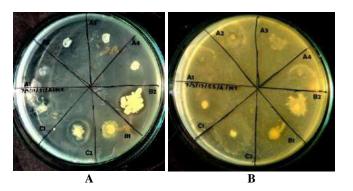


Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to *Staphylococcus aureus* (A) and *Escherichia coli* (B)

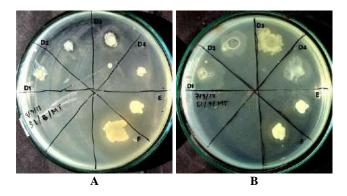


Figure 2. Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to *Staphylococcus aureus* (A) and *Escherichia coli* (B)

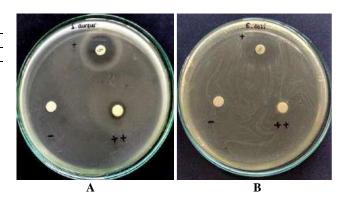


Figure 3. Results of antibiotic susceptibility test against: A. *Staphylococcus aureus* and B. *Escherichia coli*

The antibacterial properties of the supernatant produced by the symbiotic bacteria act as inhibitors against Grampositive bacteria and were merely bacteriostatic for Gramnegative bacteria. As gram-positive symbiotic bacteria widely know contain bacteriocins (Mezaini et al. 2009; Li et al. 2015) bacteriocins from Gram-positive bacteria are generally not effective against Gram-negative bacteria (Smaoui et al. 2010). Paper disc with supernatant applied to a Gram-positive bacterial plate indicate a stable clear zone even after a 48-hour incubation period. While against the Gram-negative bacteria, the presence of inhibitory activity appeared around the disc paper, but it was gradually turbulent before the incubation period reaches 24 hours. The antibacterial compounds produced by symbiont bacterial isolates showed different inhibitory activity against both tested bacteria S.aureus and E.coli. According to Soria-Mercado et al. (2011), the inner symbiotic bacteria generally have abundant populations and are specific because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria were less populated, as it required higher defense power to overcome the pathogens and predators that are around the algae.

Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Baquero and Levin 2020). Other factors that affect the inhibition potential are the concentration or intensity of antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the presence of organic matter, and the degree of acidity (pH) (Manisha and Shyamapada 2011).

The area of the symptomatic supernatant inhibition zone of *S.aureus* was 6.7 mm. According to Mounyr et al. (2016), less than 10 mm inhibition zone showed weak activity and if the inhibition zone is greater than 15 mm it indicates strong activity. Testing of antibacterial activity of the symbiont bacteria supernatant obtained was still far from the results of the antibiotic activity of the chloramphenicol control. This is because of the supernatant containing secondary metabolites. However, the test results provide clear evidence of antibacterial activity. Generally, the chemical structure of metabolites from marine products differs from the terrestrial origin. Marine bacteria are significant reservoirs of bioactive molecules that have never been found in terrestrial organisms (Barzkar et al. 2019). Seawater contains an active inhibitor agent for Gram-positive bacteria (Kapoor et al. 2017).

Identification of phenotype and genotype of symbiont bacteria

The known characteristics of symbiont bacteria through phenotypic observation and biochemical tests include rodshaped, non-acidic, non-spore-forming, non-motile, grow aerobically, negative catalase, and positive carbohydrate test Ingeneral, the selected isolate showed special characteristics possessed by lactic acid bacteria (Lactobacillus spp.), such as circular, smooth white, Grampositive colonies with brief stem cells, without shaping endospores (Davoodabadi et al. 2015).

The molecular identification was done through partial genetic analysis of 16S rDNA. PCR amplification results from the 16S region of bacterial ribosome DNA Nitrogen base sequences sorted from symbiont bacterial isolates can be seen in Figure 4. The sequencing information was under the influence of genomic information enlisted within the DDBJ/Japanese DNA Information Bank with 100% strains grouping comes about. The characters of the antibacterial strains were indistinguishable from those of Lactobacillus plantarum. The highest 100% personality, greatest score 2660, add up to score 2660, 100% inquiry scope, E esteem 0, was recorded for the taxon of adjacent microbes. The classification of the bacterial isolate is Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus plantarum.

Sequens of 16S rDNA

TGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTT GGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGGTAACGGCTCA CCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG GAATCTTCCACAATGGACGAAAAGTCTGATGGAGCAACGCCGCGCGTGAAGAAGGAGGGTTTCCGCTCGTAAAACTCTGTTGTTAAAGAAGAA CATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG GCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGC ATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGG CGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAA CGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTG AAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACAT ACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCGTCGTGTCGTGAGATGTTGG GTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAA GTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGC ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTC

Figure 4. The arrangement of nitrogen bases sequenced from symbiont bacteria, A: adenine, T: thiamine, G: guanine, C: cytosine

Data for base sequence encoding gene of 16S rDNA shows that symbiont bacteria have accurate scores for species levels with a similarity of 100% of the sequences present in GenBank. The species homology of the tested isolate was L. plantarum. L. plantarum strains separated from dairy items appeared solid antimicrobial action against the pointers strains of S. aureus, Salmonella spp, and E. coli (Hu et al. 2019). The isolation of L. plantarum from Tibetan yaks was able to restrain the development of E. coli and S. aureus (Wang et al. 2018). Some Lactobacillus strains showed antibacterial movement against Enterobacteriaceae that were safe for carbapenems (CRE). This effect may have potential applications through the utilize of the Lactobacillus strain as a starter culture in aged nourishments or as a nourishment additive to control or avoid CRE contamination (Chen et al. 2019).

In conclusion, *T. conoides* was commonly found in the gulf of Banten, Serang district, province of Banten. This research revealed that symbiont bacteria *L. plantarum* was endophytic and potentially useful as an antibacterial agent against common pathogens.

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FILE 1. BUKTI SUBMISSION / TANGGAL 7 OKTOBER 2020

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Antibacterials Potential Symbiont Bacteria of Brown Algae (Turbinaria Conoides) Obtained from Indonesian waters

Author(s) name:

Niken Dharmayanti

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Antibacterials Potential Symbiont Bacteria of Brown Algae (*Turbinaria* conoides) Obtained from Indonesian Waters

Niken Dharmayanti, Aef Permadi, Yuliati H Sipahutar, Resmi Rumenta Siregar, Arpan Nasri Siregar, Randi Bokhi Salampessy, Sujuliyani, Arma Anti

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ABSTRACT

Brown seaweed has the potential to produce bioactive compounds. It has been shown that the bacteria associated with seaweed
are involved in the production of metabolites associated with their host. Microbes may be present as a living symbiotic in association with
other algae as epiphytes or endophytes. In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were tested for
antibacterial activity. A total of 14 isolates were isolated, 6 of which came from external tissue, while 8 isolates came from internal tissue.
Through the antagonistic test, 7 isolates showed inhibitory activity against *Staphylococcuaureus* and 1 isolate showed the inhibition against
both *S.aureus* and *E.coli*. Phenotypic and genotypic identification showed that the species symbiont bacteria was *Lactobacillus plantarum*.

14 Keywords: bioassay, antagonistic, diffusion paper disc, *Lactobacillus plantarum*.

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INTRODUCTION

16 Indonesia is the largest producer of seaweed in the world (FAO 2016) cultured in nearshore coastal regions. Seaweeds produce diverse bioactive metabolites with antibacterial, antiviral, antifungal and cytotoxic properties (Zainuddin and 17 Malina, 2009 in Siregar et al., 2012). It has been shown that the bacteria associated with seaweed as epiphytes or endophytes 18 19 are involved in the production of metabolites (Sartika et al. 2014, Kalaivani et al., 2016). Symbiont bacteria isolates in 20 algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of the form of symbiotic mutualism. Algae provide needed sites and nutrients, while the bacteria encourage growth and protect the algal surface 21 22 against pathogens (Hollants et al., 2012 in Sartika et al, 2014). Seaweeds can secrete secondary metabolites with 23 antibacterial properties (Burgesset et al., 1999; Armstrong et al., 2001; Yanet et al., 2003 in Nofiani, 2005).

Here we evaluate the properties of the brown alga *Turbinaria conoides* in producing bioactive compounds including
 the inhibition of human pathogens (Kalaivani et al., 2016). *T. conoides* is a tropical marine alga widely distributed in coastal
 waters in Asia. We chose this alga following extensive trials on other common macroalgae including *Sargassum* spp. and
 Euchema cottoni.

MATERIALS AND METHODS

32 Procedures

33 Sampling

Samples of *Turbinaria sp.* (about 1 kg wet weight) were taken from Lima island (S: -6.001051; E: 106.153804)
 Samples were maintained in fresh seawater for laboratory analyses within 24 hour of collection.

36 Isolation of Symbiont Bacteria Producing Antibacterial Compounds

Epibionts were extracted from 15 grams of algae by rinsin with 30 mL of sterile sea water. The rinse water was
incubated in 30 mL of nutrient broth medium shaken at room temperature for 24 hours. Bioactive compound were extracted
by crushing 15 g of algawith a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension was incubated
with 30 mL broth nutrient medium and shaken att room temperature for 24 hours.

After extraction process, the refreshed samples in the 30 ml broth nutrient medium were diluted into 9 ml broth nutrient sterile 10⁻¹ up to 10⁻⁵. Each dilutions were grown on a plate count agar medium by incubate them at 37 °C for 2 x 24 hours. After incubating the petri dishes which were contain samples from each dilution, then the colonies bacteria from alga would appear. The colonies bacteria producing antimicrobial compounds were characterized by a clear zone around the colonies. Furthermore, the colonies with stable inhibition zones were collected by isolating them on slant agar medium, with a clear code.

47 Selection of Symbiont Bacteria Isolates Antagonistically against Pathogenic Bacteria

48 To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria, a qualitative test was 49 conducted directly by scratching round the isolates on the surface of the media that has been dispersed with test bacteria 50 (*Eschericia coli* and *Staphylococcusaureus*). Media were incubated for 48 hours at 37 °C. Each scratching round of isolates 51 was then marked by a unique code.

52 Inhibition zones were determined as those showing clear zones around the colony of simbiont bacteria isolates, for 53 both *Escheriacia coli* and *Staphylococcus aureus*. Strains that showed maximum antagonistic effect against tested pathogens 54 were identified. These were isolated and selected for further antibacterial testing by paper disc and identification of 55 phenotype and genotype.

56 Antibacterial Potential Testing of Symbiont Bacterial Isolate by Paper Disc Diffusion

57 Testing the supernatant of symbiont bacteria for inhibitory growth of E.coli and S.aureus was performed by the 58 agar diffusion method (Hudzicki, 2009)). Supernatant was obtained by separating the filtrate and supernatant bycentrifuge 59 for 1 hour (25 °C and 3000 rpm). Paper discs containing supernatant 40 µL and the negative control nutrient broth 40 µL were left for 1 hour to reduce the water excess, and positive control chloramphenicol 0.01 mg/mL, were placed on the 60 surface of the Mueller Hinton Agar medium containing 1 mL test bacteria and incubated for 48 hours at 37 °C. The 61 supernatant diffuses from the disc into the agar. If the organism is killed or inhibited by both the supernatant and 62 63 chloramphenicol as antibiotic positive control, there will be no growth in the immediate area around the disc, this is called 64 the zone of inhibition. The zone sizes were compared to assess bioactivity as sensitive, resistant, or intermediate, in each 65 case the resistance zone where shows no colonies growth was measured by using ruler to the nearest mm.

66 Identification of Phenotype and Genotype of Symbiont Bacteria

67 General bacterial identification (Lay, 1994 and identification keys from Cowan and Steel (1993)) followed colony characteristic observations on liquid medium and solid medium, observing cell morphology (gram staining, spore staining, 68 69 and Ziehl-Neelsen staining), and Biochemistry test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase). 70 The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria conoides 71 samples on the agar medium in pour plating. Observation of medium incubated with temperature 37°C was done at 72 incubation time reached 24 hours and 48 hours. The data obtained from the bacterial isolate characterization were used to 73 estimate the type of symbiotic bacteria isolated from *Turbinaria conoides*. Determination of the type of bacteria was 74 performed based on. Symbiont bacteria species was determined by molecular testing.

75 The DNA of the symbiont bacteria isolates was amplified using primers 9F and 1541R. The DNA bands used were 76 relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf German) with a first predenaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C for 30 77 seconds, primary attachment at 50 ° C for 30 seconds and extension at 72 ° C for 90 seconds. After 30 cycles completed, 78 followed by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular identification was done 79 through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989. 80 81 Modified). PCR Amplification on 16S rDNA using Primer 9 F: 5'-- AAG GAG GTG ATC CAG CC-3' and Primer 1541 82 R: 5' - GAG TTT GAT CCT GGC TCA G - 3' (White et al., 1990; O'Donnell, 1993). The analysis of nitrogen base sequence 83 readings was performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). 84 The next sequenced data trimmed and assembled raw were using the BioEdit program 85 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data 86 registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/)

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RESULTS AND DISCUSSION

88 The Result of Symbiont Bacteria Isolation

89 Samples consisted of the inside and outside of the algae, as many as 20 petri for 2 replications resulted in colonies 90 with the inhibit zone of 14 colonies, 6 of which were from epibionts, while the other 8 came from the algal tissue. The 91 results of identification of colonies grown on mixed cultures can be seen in Table 1. and identification of isolates isolated 92 into slant agar can be seen in Table 2.

93 94 95

102103 Tabel 1. Macroscopic forms of bacterial colonies

N	Colonia and			Mor	hology of colonies	
No	Colony code	Shape	Color	Edges	Elevation	
1	TUL ² -A1-2	Round	White	Flat	Convex shiny	
2	TUL ² -A2-2	Round	White	Flat	Convex shiny	
3	TUL ² -A3-2	Round	White	Flat	Convex shiny	
4	TUL ² -A4-2	Round	White	Flat	Convex shiny	
5	TUL ² -B1-2	Round	White	Crooked	Convex shiny	
6	TUL ² -B2-2	Round	White	Crooked	Convex shiny	
7	TUD ⁴ -C1-2	Round	White	Flat	Convex shiny	
8	TUD ⁴ -C2-2	Round	White	Flat	Convex shiny	
9	TUD ² -D1-2	Round	White	Crooked	Convex shiny	
10	TUD ² -D2-2	Round	White	Crooked	Convex shiny	
11	TUD ² -D3-2	Round	White	Crooked	Convex shiny	
12	TUD ² -D4-2	Round	White	Crooked	Convex shiny	
13	TUD ⁵ -E-2	Round	White	Flat	Convex shiny	
14	TUD ³ -F-2	Round	White	Flat	Convex shiny	

104 Information:

105 *The code of isolates TUL/TUD states the isolates originating from the outer/inner algae

106 ** The code of isolates $\binom{2}{4}$, $\binom{4}{5}$, $\binom{3}{3}$ states isolates obtained from the dilution

107 *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed on the plate

110 **** The code of number 2 identifies the isolate obtained from the second repeat

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112 Table 2. Identification of the isolates on slant agar

No	Code of isolates		Solid medium
INO	Code of isolates	Shape	Color
1.	TUL ² -A1-2	Spread	Milky white
2.	TUL ² -A2-2	Spread	Milky white
3.	TUL ² -A3-2	Spread	Milky white
4.	TUL ² -A4-2	Spread	Milky white
5.	TUL ² -B1-2	Rhizoidal	Cloudy white
6.	TUL ² -B2-2	Rhizoidal	Cloudy white
7.	TUD ⁴ -C1-2	Spread	Milky white
8.	TUD ⁴ -C2-2	Spread	Milky white
9.	TUD ² -D1-2	Rhizoidal	Cloudy white
10.	TUD ² -D2-2	Rhizoidal	Cloudy white
11.	TUD ² -D3-2	Rhizoidal	Cloudy white
12.	TUD ² -D4-2	Rhizoidal	Cloudy white
13.	TUD ⁵ -E-2	Spread	Milky white
14.	TUD ³ -F-2	Spread	Milky white

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Bacteria is isolated into a solid medium, then there is a group commonly referred to as a colony. The colony's shape is different for each species and it is characteristic of a particular species (Dwidjoseputro, 1981).

116 The Selection Results Symbiont Bacteria Producing Antibacterial Compounds117

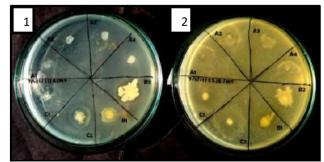


Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to S.aureus (1) and E.coli (2)

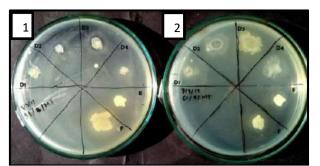


Figure 2. Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to S.aureus (1) and E.coli (2)

Based on the results of the direct challenge test, 7 bacterial isolates from 14 isolates tested showed inhibitory activity against *S.aureus* and only 2 of the 7 isolates had inhibitory activity against *E.coli*. The isolate codes that have inhibitory zones against *S.aureus* bacteria are TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2, whereas TUD4-C1-2, And TUD4-C2-2 showed inhibition zones against both pathogenic bacteria being tested but the inhibitory activity against *E.coli* was not as good as its inhibition against *S.aureus*.

128 Isolates with showing inhibition were re-selected by looking at the best and largest clear zone. Isolates with code 129 TUD4-C2-2 were isolates which had the best inhibition zone. Bacterial isolates derived from tissue showed better inhibition 130 than isolates derived from epibionts. Inhibitory zone and diameter measurement results against S.aureus and E.coli can 131 be seen in Figure 3 and Table 3. Positive controls have a broader spectrum in inhibiting both types of test bacteria with 16.8 132 mm inhibition against S.aureus and 13.8 mm against E.coli. Chloramphenicol with a concentration of 0.03 mg on a paper 133 disc is highly active if its inhibition zone is more than 18 mm (Lay, 1994), while the dose of chloramphenicol (positive control) used is lower at 0.01 mg, so it can be said that bacteria Test is sensitive to positive control. Negative control (NB 134 without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that a 135 136 supernatant still containing medium has no effect on the activity formed. From the stability of the measured inhibition zone, 137 the antibacterial properties of the supernatant produced by the symbiotic bacteria act as inhibitors against Gram positive 138 bacteria and are merely bacteriostatic for Gram negative bacteria. Paper disc with a supernatant applied to a Gram positive 139 bacterial plate indicates a stable clear zone even after a 48-hour incubation period. While against Gram negative bacteria. 140 around the disc paper shows the presence of inhibitory activity but gradually become turbid before the incubation period 141 reaches 24 hours.

142The sequence of DNA sequencing results of the 16S rDNA encoding of symbiont bacteria isolates showed an accurate143score for species level with a similarity of \geq 99% of the sequences present in GenBank, Then the species homology of the144isolates tested was Lactobacillus plantarum. Classification of bacterial isolates are Bacteria; Firmicutes; Bacilli;145Lactobacillales; Lactobacillus; Lactobacillus plantarum.

The antibacterial compounds produced by symbiont bacteria isolates showed different inhibitory activity against both tested bacteria *S.aureus* and *E.coli*. According to Abubakar et al (2011) in Sartika et al (2014) the inner symbiotic bacteria generally have abundant populations and are specific microbes because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria originating from the surface have a population that is less suspected because it requires higher defense power to overcome the pathogens and predators that are around the algae.



Figure 3. Results of antibiotic susceptibility test against S.aureus and E.coli

Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Lay, 164 1994). Other factors that influence the ability of inhibition are the concentration or intensity of antimicrobial agents, the 165 number of microorganisms, the temperature, the species of microorganisms, the presence of organic matter and the degree 166 of acidity (pH) (Sulistijowati and Mile, 2015).

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120 121

168 Table 3. Results of measurement of inhibitory zone diameter of antibacterial compounds

			Diameter of zone	e inhibition (mm)			
		Gram positive			Gram negative		
Repetition	Symbiont bacterial (++)	Control (+)	Control (-)	Symbiont bacterial (++)	Control (+)	Control (-)	
1	5,5	16	0	0	13,5	0	
2	7,8	17,5	0	0	14	0	
Average	6,7	16,8	0	0	13,8	0	

169 The area of the symptomatic supernatant inhibition zone of S.aureus was 6.7 mm. According to Edrada (1998) in Kusumadewi (2004) a measured inhibition zone of less than 10 mm shows weak activity and strong activity if the the 170 inhibition zone is greater than 15 mm. Testing of antibacterial activity of the symbiont bacteria supernatant obtained was 171 still far from the results of the antibiotic activity of the chloramphenicol control. This is because the antibacterial compound 172 of the extracted symbiont bacteria was a supernatant containing secondary metabolites. However, the test results provide 173 clear evidence of antibacterial activity. Generally the chemical structure of metabolites from marine products differs from 174 175 those of terrestrial origin (Gudbjarnason 1999 in Nofiani, 2005). Seawater contains an active inhibitor agent for Gram 176 positive bacteria Okami (1982) in Nofiani (2005)

177 Identification of Phenotype and Genotype of Symbiont Bacteria

178 Based on phenotypic identification results through cell staining and biochemical testing, symbiont bacteria were rod 179 shaped, non acidic, non spore forming, non motile, aerobically grown, negative catalase, and positive to carbohydrate tests. In general, the identification of selected isolates showed specific characteristics of lactic acid bacteria (*Lactobacillus* spp.), 180 181 such as round colonies, milky white, Gram positive with short stem cells, without forming endospores (Desniar 2012 in 182 Saskia, 2014).

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Lactobacillus plantarum_100%

TTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGGATAACACCTGGAAACAGATGCTAATACCG CATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGCGTATTAGCTAGATG GTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAA GGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTA AAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTC CATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTC GAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCT TCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCG CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTA GACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG AGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG CTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGC ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTC

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Figure 4. Sequens of 16S rDNA

Data for base sequence encoding gene of 16S rDNA shows that symbiont bacteria has accurate scores for species 186 levels with a similarity 100% of the sequences present in GenBank (Figure 4), The species homology of the tested isolate 188 was Lactobacillus plantarum.

CONCLUSION

Turbinaria conoides is commonly found in the gulf of Banten, Serang district, province of Banten. This research shows that symbiont bacteria Lactobacillus plantarum are endophytic and potentially useful as an antibacterial agent against common pathogens.

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FILE 2. BUKTI ACCEPTED / TANGGAL 26 DESEMBER 2020

Antibacterial potential of symbiont bacteria of brown algae (*Turbinaria conoides*) obtained from Indonesian waters

NIKEN DHARMAYANTI", ARMA ANTI, RESMI RUMENTA SIREGAR, YULIATI H. SIPAHUTAR, AEF PERMADI, ARPAN NASRI SIREGAR, RANDI BOKHI SALAMPESSY, SUJULIYANI, SITI ZACHRO NURBANI, HENI BUDI PURNAMASARI

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Manuscript received: 7 October 2020. Revision accepted: 26 December 2020.

Abstract. Dharmayanti N, Anti A, Siregar RR, Sipahutar Y, Permadi A, Siregar AN, Salampessy RB, Sujuliyanti, Nurbani SZ, Purnamasari HB. 2020. Title. Biodiversitas 22: 373-378. Brown seaweeds have the potential to produce bioactive compounds. Bacteria associated with seaweeds are involved in the production of metabolites. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes. In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were tested for antibacterial activity. A total of 14 bacteria were isolated, of which 6 were isolated from external tissue, while 8 from internal tissue. Results of an antagonistic test revealed that 7 isolates showed inhibitory activity against *Staphylococcus aureus* and only 1 isolate showed the inhibition against both *S. aureus* and *Escherichia coli*. Phenotypic and genotypic analysis showed that the symbiont bacteria was *Lactobacillus plantarum*.

Keywords: Bioassay, brown seaweed, antagonistic, diffusion paper disc, Lactobacillus plantarum

INTRODUCTION

Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse, and unique compounds (Bahare et al. 2019). Many substances are obtained from seaweed, such as alginates. carrageenan, and agar, which have been used for decades in raditional medicine, pharmacology, and food (Andrea et 2019). Other compounds have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory, and antifouling activity. Therefore, seaweed can provide promising bioactive that can be used in the treatment of human diseases or new antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Seaweed applications are particularly used in the design of new antimicrobial drugs. Research for the identification of promising algal species, standardization of analytical methods, isolation of compounds through integrated fractionation of bioassays, detailed chemical characterization and evaluation of their safety, evaluation of synergistic effects between components, and efforts to improve yields and lowering extraction costs is needed (Marie et al. 2016). In later decades, e strides microbiological procedures have altogether made a difference in build-up phylogenetic

affiliations of seaweed-related epi bacterial communities and endophytes. Be that as it may, there's inadequately prove that utilitarian connections for seaweed-bacterial intuitive can be built up and well caught on. Epiphytic bacterial communities are rapid colonizers of the ocean growth surface, some of the time versatile and able to quickly metabolize algal exudates (Singh and Reddy, 2014). It has traditionally been used for children's fever, as a fertilizer, repellent, including antioxidant, antiinflammatory, antimicrobial, and anti-cancer (Gupta et al. 2011). Seaweeds can secrete secondary metabolites with antibacterial properties (Emer and Nissreen 2016). The symbiotic mutualism occurs as algae provide essential sites and nutrients, while the bacteria encourage growth and protect the algal surface against symbiont bacteria isolates as algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of infections acquired from the community (Arumugama et al. 2017). T. conoides is a tropical marine alga widely distributed in coastal waters in Asia. This study evaluates the properties of the brown alga Turbinaria conoides in producing bioactive compounds including the inhibition of human pathogens.

MATERIALS AND METHODS

Procedures

Sampling

Samples of Turbinaria sp. (about 1 kg wet weight) were taken from Lima island (S: -6.001051; E: 106.153804) Samples were maintained in fresh seawater for laboratory analysis within 24 hours of collection.

Isolation of symbiont bacteria producing antibacterial compounds

Bacteria were isolated in a solid medium and the size of the colony was different for each species and was characteristic of a particular species (Erin 2012). Epibionts were extracted from 15 grams of algae by rinse in with 30 mL of sterile seawater. The rinse water was incubated in 30 mL of nutrient broth medium and shaken at room temperature for 24 hours. The bioactive compound was extracted by crushing 15 g of alga with a mortar and pestle with the addition of 15 ml of sterile seawater. The suspension was insert into a 30 ml nutrient broth medium and shaken at room temperature for 24 hours.

. After the extraction process, 1 ml of refresh samples were diluted in a 9 ml of sterile nutrient broth to make 10^{-1} dilution. This process was continued to achieve 10^{-4} dilution Each dilution was grown on a plate count agar medium by incubating them at 37 °C for 2 x 24 hours.. Colonies of bacteria that produce antimicrobial compounds were characterized by a clear zone. Furthermore, the colonies with stable inhibition zones were collected by solating them on a slant agar medium.

Selection of symbiont bacteria isolates antagonistically against pathogenic bacteria

For this, a qualitative test was carried out directly by scratching the isolates on the surface of the media that has been dispersed with two test bacteria, i.e. Escherichia coli and Staphylococcus aureus (Monte, et al 2014)). The media was then incubated for 48 hours at 37 °C. Each scratching round of isolates was then marked by a unique code. Inhibition zones were showing clear zones around the colony of symbiont bacteria isolates for both Escherichia coli and Staphylococcus aureus. Strains that showed maximum antagonistic effect against tested pathogens were identified. Strains showing maximum antagonistic effects were isolated and selected for antibacterial testing by the paper disc diffusion method. Further, the strains were identified at the phenotypic and genotypic levels.

Antibacterial potential testing of symbiont bacterial isolate by paper disc diffusion

Antibacterial testing of symbiont bacteria for inhibitory growth of E. coli and S. aureus was performed by the paper disc diffusion method (Grela et al. 2018). The supernatant was obtained by separating the filtrate and the supernatant was centrifuged for 1 hour (25 °C and 3000 rpm). Paper discs containing 40 µL supernatant was considered as the treatment while 40 µL nutrient broth was used in negative control and chloramphenicol (0.01 mg/mL) was used as a positive control. After that, the discs were placed on the

surface of the Mueller Hinton Agar medium containing mL test bacteria and incubated for 48 hours at 37 °C. The supernatant diffuses from the disc into the agar. The presence of a clear zone around the supernatant and antibiotic discs was measured by the meter rule in mm. The zone sizes were compared to assess bioactivity as sensitive, resistant, or intermediate, and the presence of a clear zone was measured by the meter rule in mm.

Identification of phenotype and genotype of symbiont bacteria

General bacterial identification based on colony characteristic observations on liquid medium and solid medium followed by cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and biochemical test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase) as described by Phumudzo, (2013). The initial selection of isolates from mixed cultures was carried out after enrichment and planting of Turbinaria conoides samples on the agar medium. The plates were incubated at 37°C temperature for 24 to 48 hours. The data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from Turbinaria conoides. The DNA of the symbiont bacteria isolated was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf German) with a first predenaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95 ° C for 30 seconds, primary attachment at 50 ° C for 30 seconds, and extension at 72 ° C for 90 seconds, followed by the elongation phase at 72 ° C for 5 min and cooling at 4 ° C for 20 min. Molecular identification was done through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al., 1989 Modified). 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 performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The next sequenced raw data were trimmed and assembled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data registered in DDBJ / DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/).

RESULTS AND DISCUSSION

The result of symbiont bacteria isolation

A total of 14 colonies were isolated, of which 6 were from epibionts while the other 8 were from algal tissue. The macroscopic results of colonies on mixed cultures can be seen in Table 1, and on slant agar can be seen in Table 2.

The selection results symbiont bacteria producing antibacterial compounds

Based on the results of the direct challenge test, only 5 pacterial isolates i.e. TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2 showed inhibitory activity

Tabel 1. Macroscopic forms of bacterial colonies.

Colony	Morphology of colonies			onies
code	Shape	Color	Edges	Elevation
TUL ² -A1-2	Round	White	Flat	Convex shiny
TUL ² -A2-2	Round	White	Flat	Convex shiny
TUL ² -A3-2	Round	White	Flat	Convex shiny
TUL ² -A4-2	Round	White	Flat	Convex shiny
TUL ² -B1-2	Round	White	Crooked	Convex shiny
TUL ² -B2-2	Round	White	Crooked	Convex shiny
TUD ⁴ -C1-2	Round	White	Flat	Convex shiny
TUD ⁴ -C2-2	Round	White	Flat	Convex shiny
TUD ² -D1-2	Round	White	Crooked	Convex shiny
TUD ² -D2-2	Round	White	Crooked	Convex shiny
TUD ² -D3-2	Round	White	Crooked	Convex shiny
TUD ² -D4-2	Round	White	Crooked	Convex shiny
TUD ⁵ -E-2	Round	White	Flat	Convex shiny
TUD ³ -F-2	Round	White	Flat	Convex shiny

Note: *The code of isolates TUL/TUD states the isolates originating from the outer/inner algae. ** The code of isolates $(^2)$, $(^4)$, $(^5)$, $(^3)$ states isolates obtained from the dilution. *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed on the plate. **** The code of number 2 identifies the isolate obtained from the second repeat

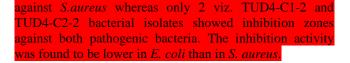


Table 2. Macroscopic form of the isolates on slant agar

Call offerlater	Solid	Solid medium		
Code of isolates	Shape	Color		
TUL ² -A1-2	Spread	Milky white		
TUL ² -A2-2	Spread	Milky white		
TUL ² -A3-2	Spread	Milky white		
TUL ² -A4-2	Spread	Milky white		
TUL ² -B1-2	Rhizoidal	Cloudy white		
TUL ² -B2-2	Rhizoidal	Cloudy white		
TUD ⁴ -C1-2	Spread	Milky white		
TUD ⁴ -C2-2	Spread	Milky white		
TUD ² -D1-2	Rhizoidal	Cloudy white		
TUD ² -D2-2	Rhizoidal	Cloudy white		
TUD ² -D3-2	Rhizoidal	Cloudy white		
TUD ² -D4-2	Rhizoidal	Cloudy white		
TUD ⁵ -E-2	Spread	Milky white		
TUD ³ -F-2	Spread	Milky white		

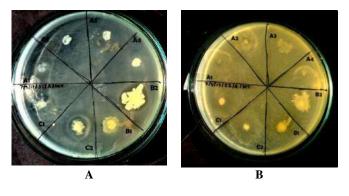


Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to *S.aureus* (A) and *E.coli* (B)

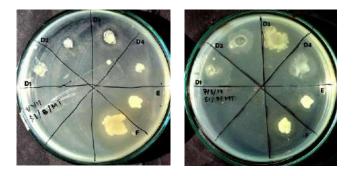


Figure 2. Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to *S.aureus* (A) and *E.coli* (B)

B

A

Isolates with code TUD4-C2-2 had the best inhibition zone. Bacterial isolates derived from algal tissue showed better inhibition than isolates derived from epibionts. The inhibitory zone and diameter measurement results against S. aureus and E. coli can be seen in Figure 3 and Table 3 Positive controls showed 16.8 mm inhibition zone against S. aureus and 13 8 mm inhibition zone against E. coli. Chloramphenicol with a concentration of 0.03 mg on a paper disc is highly active if its inhibition zone is more han 18 mm (Mounyr et al., 2016), while the dose of chloramphenicol (positive control) used was less than 0.01 mg, so it can be said that bacteria test was found to be sensitive to the positive control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that supernatant does not affect the activity formed. The antibacterial properties of the supernatant produced by the symbiotic bacteria act as inhibitors against Gram-positive bacteria and were merely bacteriostatic for Gram-negative bacteria. As gram-positive symbiotic bacteria widely know contain bacteriocins (Mezaini A et al, 2009 and Li D. Et al, 2015) bacteriocins from Grampositive bacteria are generally not effective against Gramnegative bacteria (Smaoui et al, 2010). Paper disc with supernatant applied to a Gram-positive bacterial plate indicate a stable clear zone even after a 48-hour incubation period. While against the Gram-negative bacteria, the presence of inhibitory activity appeared around the disc paper, but it was gradually turbulent before the incubation period reaches 24 hours. The antibacterial compounds produced by symbiont bacterial isolates showed different inhibitory activity against both tested bacteria S. aureus and E.coli. According to Irma et al. (2011), the inner symbiotic pacteria generally have abundant populations and are specific because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria were less populated, as it required higher defense power to overcome the pathogens and predators that are around the algae.

Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Fernando and Bruce, 2020). Other factors that affect the inhibition potential are the concentration or intensity of antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the presence of organic matter, and the degree of acidity (pH) (Manisha and Shyamapada 2011).

The area of the symptomatic supernatant inhibition zone of *S.aureus* was 6.7 mm. According to Mounyr et al (2016), less than 10 mm inhibition zone showed weak activity and if the inhibition zone is greater than 15 mm it indicates strong activity. Testing of antibacterial activity of the symbiont bacteria supernatant obtained was still far from the results of the antibiotic activity of the chloramphenicol control. This is because of the supernatant containing secondary metabolites. However, the test results provide clear evidence of antibacterial activity. Generally, the chemical structure of metabolites from marine products differs from the terrestrial origin. Marine bacteria are significant reservoirs of bioactive molecules that have never been found in terrestrial organisms (Giovanna, 2020). Seawater contains an active inhibitor agent for Grampositive bacteria (Kapoor et al. 2017).

Identification of phenotype and genotype of symbiont bacteria

The known characteristics of symbiont bacteria through phenotypic observation and biochemical tests include rodshaped, non-acidic, non-spore-forming, non-motile, grow aerobically, negative catalase, and positive carbohydrate test In general, the selected isolate showed special characteristics possessed by lactic acid bacteria (Lactobacillus spp.), such as circular, smooth white, Grampositive colonies with brief stem cells, without shaping endospores (Davoodabadi et al. 2015).

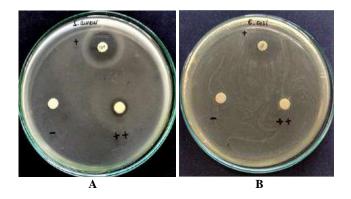


Figure 3. Results of antibiotic susceptibility test against: A. *Staphylococcus aureus* and B. *Escherichia coli*

		The	diameter of zoi	ne inhibition (mm)		
Repetition -	Gram	ram-positive		Gram-negative		
Kepetition -	Symbiont bacterial	Control	Control	Symbiont bacterial	Control	Control
	(++)	(+)	(-)	(++)	(+)	(-)
1	5.5	16	0	0	13.5	0
2	7.8	17.5	0	0	14	0
Average	6.7	16.8	0	0	13.8	0

Table 3. Results of inhibitory zone diameter

Sequens of 16S rDNA

Figure 4. The arrangement of nitrogen bases sequenced from symbiont bacteria, A = adenine, T = thiamine, G = guanine, C = cytosine.

The molecular identification was done through partial genetic analysis of 16S rDNA. PCR amplification results from the 16S region of bacterial ribosome DNA Nitrogen base sequences sorted from symbiont bacterial isolates can be seen in Figure 4. The sequencing information was under the influence of genomic information enlisted within the DDBJ / Japanese DNA Information Bank with 100% strains grouping comes about. The characters of the antibacterial strains were indistinguishable from those of *Lactobacillus plantarum*. The highest 100% personality, greatest score 2660, add up to score 2660, 100% inquiry scope, E esteem 0, was recorded for the taxon of adjacent microbes. The classification of the bacterial isolate is Bacteria; Firmicutes; Bacilli; Lactobacillaes: Lactobacillus plantarum.

Data for base sequence encoding gene of 16S rDNA shows that symbiont bacteria have accurate scores for species levels with a similarity of 100% of the sequences present in GenBank. The species homology of the tested isolate was Lactobacillus plantarum. Lactobacillus plantarum strains separated from dairy items appeared solid antimicrobial action against the pointers strains of Staphylococcus aureus, Salmonella spp, and Escherichia coli (Hu et al 2019). The isolation of L. plantarum from Tibetan yaks was able to restrain the development of E coli and S. aureus (Wang et al 2018). Some LLactobacillus strains showed antibacterial movement Enterobacteriaceae that were safe for carbapenems (CRE) This effect may have potential applications through the utilize of the Lactobacillus strain as a starter culture n aged nourishments or as a nourishment additive to control or avoid CRE contamination (Chen et al 2019).

In conclusion, *Turbinaria conoides* was commonly found in the gulf of Banten, Serang district, province of Banten. This research revealed that symbiont bacteria *Lactobacillus plantarum* was endophytic and potentially useful as an antibacterial agent against common pathogens.

ACKNOWLEDGEMENTS

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FILE 3. PERTANYAAN/PERINTAH PERBAIKAN DARI PARA REVIEWER JURNAL

3. file pertanyaan/perintah perbaikan dari para reviewer jurnal (bisa beberapa kali, lengkap dengan informasi tanggal) serta jawaban dari pengarang korespondensi Agar klarifikasi disusun secara sistematis, seperti perintah di atas.

Editor/	Date	Reviewer Suggestion	Author response
Reviewer Editor	9-10-2020	 Dear author, Thank you very much for your manuscript submission. Unfortunately, your manuscript does not meet our requirements: At least, to published in the Biodiversitas journal, you need to compose a minimum of 20 references which 80% of international scientific journals published in the last 10 years (2010-2020), and maximum 10% references in the local language (not English). And please write the references based on the author's guidelines. This manuscript is too brief to be published in the Biodiversitas journal. At least, you need to compose a 2000 words article from the introduction to a conclusion (table and figure are excluded). Kindly check and correct accordingly 	The number of references has been added to 27 libraries which originally amounted to 13 libraries. The number of 27 libraries has exceeded the minimum limit of 20 libraries. Literature composition consists of international journals of science for the last 10 years (2010-2020) as many as 23 libraries or 85% and 0% of local languages meet a minimum of 10% of local languages (not English) The number of words has been added to 2208 words outside Tables and Figures that have exceeded the minimum limit of 2000 words The addition of these words is done by developing a discussion using reference references.
Reviewer B:	9-11-2020	Dear authors of the manuscript: Antibacterial potential of symbiont bacteria of brown algae (Turbinaria conoides) obtained from Indonesian waters. The manuscript focus on symbionts from this macroalga, which may present antibacterial properties. It was with pleasure that I read the work. The study seems relevant, interesting, and important. Nowadays the research of new antimicrobial compounds is crucial and it is known that the sea is a reservoir of this kind of compound. Although I think the outputs selected for the study were	The paper has been improved as requested by adding a discussion of both macroalgae and symbiotic bacteria using libraries from several recent journals

		relevant, I believe you could be more audacious and	
		made other assays to prove antimicrobial activity. I	
		would also like to see results using the macroalgae itself	
		since it is also a pool antioxidant and antimicrobial	
		compounds. Even so, is interesting the symbiotic	
		bacteria that you have found. The results could be	
		better presented and the quality should be improved, or	
		presented in a different way. In attach, I send the	
		manuscript with minor comments for the authors'	
D. 1	0.44.0000	consideration. Recommendation: Resubmit for Review	
Reviewer F:	9-11-2020	The paper in question concerns itself with isolating	The paper has made improvements as requested
		bacterial symbiotes of T. conoides and studying their	by changing reference sources and discussions.
		antibacterial activity. In my opinion, the work itself is	Writing improvements have been made both
		performed on an acceptable level, but the paper	lines 31-32 as well as lines 28.
		requires some corrections: 1. The text needs careful	Figure 4 has been replaced not in the form of an
		editing. For example, lines 31-32 go like this: Turbinaria	image but returned to the DNA chain
		conoides belongs to the family of The recent scientific	arrangement according to the sequencing results.
		trends target the pursuit for phytochemicals from	The proposed references have been adjusted for
		marine algae due to their numerous health-promoting	discussion.
		effects, pathogens (Mark LW et al. 2016). Obviously, the	
		alga in question does not belong to the family of recent	
		scientific trends, it belongs to the family Sargassaceae.	
		This part was probably copypasted incorrectly, and "due	
		to" is in bold for no apparent reasons. There are other	
		typos and similar issues, eg line 28 includes a mention	
		of "bacteri associated with seaweed", while it should be	
		"bacteria". 2. The sequence of antibacterial strain is	
		only provided as an image. I would prefer it to be	
		deposited to Genbank/DDBJ or, at the very least,	
		included as plaintext in the paper. The screenshot in fig.	
		4 does not permit copying sequence for some analysis	
		the readers might want to run. Even despite 100%	
		identity to other published sequences, the fact that the	
		strain from Lima island is identical to those isolated	

		from elsewhere may be important for someone interested in the distribution and diversity of Lactobacillus. 3. Lactobacillus plantarum is well-known for its antibacterial activity, see eg: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC65933 89/ https://www.sciencedirect.com/science/article/abs/pii/ S0882401017314559 https://www.frontiersin.org/articles/10.3389/fmicb.201	
		9.00789/full I think the dicussion and conclusions would benefit from referencing this fact. Should these issues be fixed, I believe the paper will be acceptable for publication. Recommendation: Revisions Required	
Reviewer A:	11-12-2022	 You did not mention direct test in the method but you wrote it your results. You wrote the results but you did not discuss it with other results add some references. You isolated 14 isolates. Were they all from <i>Lactobacillus</i>. Write references as per the rule of journal norm there are so many mistakes. Some references are missing in the reference so please check it. 	 Paper has been improved as follows: 1. In the methods section, it is clear about how to te the results in the Results and discussion section. 2. In the results and discussion section, discussion h including the results of other studies based on ref 10 years (2010 – 2020). 3. The authors obtained 14 isolates of symbiont bac one symbiont with the best inhibitory zone agains coli bacteria to be tested for DNA sequencing and sequencing results as Lactobacillus plantarum. 4. Bibliography writing has been improved according writing a Library (APA). 5. Already fixed missing Library writes,