Antibacterial Activity and Molecular Identification of Soft Coral *Sinularia* sp. Symbiont Bacteria from Karimunjawa Island against Skin Pathogens *Propionibacterium acnes* and *Staphylococcus epidermidis*

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Abstract. Soft corals can produce bioactive compounds that act as antibacterials. *Sinularia* sp. is a soft coral that can produce antibacterial compounds. Symbion bacteria are bacteria that can produce bioactive compounds that tend to be the same as their host. The use of symbiont bacteria can be an alternative to exploit the potential of *Sinularia* sp. without harming marine life. This study aims to determine the antibacterial potential of the symbiont bacteria obtained from the soft coral *Sinularia* sp. in inhibiting the growth of *Propionibacterium acnes* and *Staphylococcus epidermidis*. The antibacterial activity test was carried out by the agar diffusion method using disc paper. Antibacterial activity was indicated by the formation of a clear zone around the disc paper. The results showed that LA7 isolate had the best antibacterial activity with an average clear zone size of 14.6 mm against *Staphylococcus epidermidis* and 16.6 mm against *Propionibacterium acnes*. LA7 isolates identified species based on the 16S rRNA gene with primers 27F and 1492R. Based on molecular species support, it was found that LA7 isolate is *Bacillus aerius* with a homology level of 98.17%.

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1 Introduction

Acne problems are experienced by more than 80% of the population aged 12-44 years. Generally, acne occurs during puberty (8-9 years) when the production of androgen hormones increases dramatically and results in increased secretion of sebum keratin [1]. Acne (acne vulgaris) is a human skin disease in the form of inflammation caused by oil accumulation in the polysebaceous glands, causing the growth of acne-causing bacteria [2] characterized by the presence of comedones, papules, pustules, nodules, cysts, and scars [3]. Bacteria that cause acne consist of *Staphylococcus epidermidis* [4], *Staphylococcus aureus* [5], and *Propionibacterium acnes* [6]. Acne treatment methods can be done by suppressing the growth of acne-causing bacteria and reducing inflammation in acne. The biodiversity that exists in Indonesia, especially in the oceans, can certainly be explored to obtain bioactive compounds that can be used for the treatment of acne.

Indonesia is a maritime country with a very abundant diversity of marine life. One of the many types of marine life is coral reefs. Coral reefs are marine ecosystems where marine biota live with ecological, economic, tourist, chemical and biological functions [7]. One of the biotas that inhabit coral reefs is soft coral. Tanod et al. [8] stated that soft corals (*phylum Cnidaria*) are invertebrate animals that live in coral reef ecosystems. Soft corals (*Octocorallia, Alcyonacea*) are included in the Coelenterata animals that live in tropical and subtropical shallow waters [9]. According to statistical data from the Karimunjawa National Park Agency for 2019, there are 23 species of soft corals whose potential has not been explored in biotechnology.

Soft corals produce bioactive compounds as a means of self-protection [10]. Bioactive compounds produced by soft corals can act as antibacterials in the form of steroids, terpenoids, and steroid glycosides [11]. *Sinularia* sp. produce bioactive compounds with antibacterial properties [7;9]. *Sinularia* sp. also has antiviral activity [12], anti-inflammatory and anticancer [13], NFkB and iNOS inhibitors [14] and NO inhibitors [15, 16]. To facilitate the multiplication of bioactive compounds from *Sinularia* sp. This can be done with the help of the symbiont bacteria from this soft coral. Symbion bacteria are organisms that attach and interact with the host without causing negative impacts. The term bacterial symbiont was originally introduced by Frank, Van Beneden, and De Bary in 1870 [17]. Mutualistic symbiotic relationship between *Sinularia* sp. with its symbiont bacteria, *Sinularia* sp. provide a place to live and protect the symbiont bacteria, while the symbiont bacteria help the process of nutrient cycling and as a source of secondary metabolites [18]. The bioactive compounds produced by the symbiont bacteria are the same as their host [19]. Symbion bacteria have a short life cycle so that the production of bioactive compounds can be done quickly without requiring large areas of land.

The bioactive compounds produced by *Sinularia* sp. in the form of potential as anti-inflammatory and antibacterial, allowing this soft coral to be used in the treatment of acne. Symbion bacteria from *Sinularia* sp. can produce the same bioactive compounds as those produced by the soft corals and will assist in the production of bioactive compounds. Therefore, this study will explore the potential of the soft coral symbiont bacteria *Sinularia* sp. as from karimunjawa as an antibacterial against *P. acnes* and *S. epidermidis*. This study will also carry out qualitative tests of bioactive compounds (flavonoids, alkaloids, steroids, terpenoids, phenolics) and molecular identification of isolates that have the potential to have antibacterial activity.

2 Methods

2.1 Sampling

Sampling was carried out in Karimunjawa, Jepara, Central Java with a purposive sampling method, namely determining the location by observing the presence of soft corals by means of scuba diving. Soft coral samples that have been taken, stored in a container filled with sterile sea water.

2.2 Soft coral identification

Identification of soft coral species was carried out with the help of Soft Coral and Sea Fans books supported by statistical data from the Karimunjawa National Park Office for 2019.

2.3 Isolation of soft coral symbiont bacteria

Isolation of symbiont bacteria begins by taking 3 grams of soft coral polyps. The sample surface was cleaned using sterile sea water. The soft corals were crushed with sterile mortar. Soft corals that have been mashed are taken as much as 1 gram and dissolved in 9 ml of distilled water for a 10-1 dilution. Next, 1 ml was taken from the first tube and put in 9 ml of sterile distilled water for a 10-2 dilution. Sample dilution was carried out up to 10-8 dilution. At dilutions 10-6, 10-7, 10-8, 1 ml was taken using a micropipette and inoculated into a petri dish with marine agar media using the spread plate method [20]. The purification of the symbiont bacteria was carried out using the streak plate method with the help of round loops [21]. The pure bacterial culture was transferred to the agar slanting media for marine agar in a test tube as culture stock and working culture. Culture maintenance is carried out by rejuvenating isolates every four weeks [22].

2.4 Characterization of soft coral symbiont bacterial isolate

Observation of macroscopic morphology was carried out by observing bacterial colonies. Colony observations included colony shape, colony colour, colony surface, margins, and elevation.

Microscopic morphological observations were carried out using the Gram stain method. The purpose of Gram staining is to see the shape of the bacterial cell and to distinguish between Gram positive and Gram-negative bacteria. The Gram stain procedure begins with sterilizing the slide using 70% alcohol. The glass object was given drops of distilled water and one ose of bacterial isolates, then mixed and fixed on a spirit lamp. Crystal violet dye (Gram A) was dropped on the fixation result and left for 1 minute then rinsed with distilled water. Lugol's solution (Gram B) was dripped onto the preparation, left for 1 minute and then rinsed with distilled water. 96% alcohol (Gram C) was dripped onto the preparation, left for 30 seconds and then rinsed with distilled water. Safranin (Gram D) was dripped onto the preparation, left for 1 minute and then rinsed with distilled water. Stained preparations were observed under a microscope and could be clarified with the addition of immersion oil [23].

2.5 Antibacterial activity test with kirby baueur method

The antibacterial test was carried out using the Kirby Bauer method, namely the agar diffusion method with paper discs. The test was carried out by growing the test bacteria on MHA media. The test bacterial suspension was inoculated by means of a swab on MHA media. Paper discs (6mm) were dripped with 20µL of a suspension of soft coral symbiont bacteria, chloramphenicol, and sterile distilled water. Chloramphenicol as a positive control and sterile distilled water as a negative control. Paper discs that have been dripped are placed on MHA media which has been overgrown with test bacteria [24]. Then incubated for 48 hours at 28°C. Antibacterial activity was observed by looking at the clear zone or inhibition zone that was formed [25].

2.6 Analysis of antibacterial compounds

Analysis of bioactive compounds using the Gas Chromatography Mass Spectrometer (GC-MS) method. The tools used are GC/MS Thermo Scientific with Trace ISQ7000 MS spec and Trace 1310 GC. The column used was Agilent 190915-433UI: 0236716H HP-5MS UI with a size of 30 m x 250 µm x 0.25 µm. The carrier gas used is helium gas. The temperature used is 100°C with an average increase of 10°C/minute and the maximum temperature is 300°C. Column flow rate 1 ml/min.

2.7 Molecular identification

Bacterial isolates with the best antibacterial activity and producing bioactive compounds were identified molecularly. Molecular identification using 16S rRNA gene markers was carried out based on the method of Noer [26] and Nafia [27] with modifications. DNA extraction was carried out using the InstaGeneTM Matrix kit protocol. DNA amplification using the polymerase chain reaction method with the help of a thermocycler. Amplification of the bacterial genomic DNA against the 16S rRNA gene was performed using a forward primer 27f (5'- AGA GTT TGA TCA CTG GCT CAG - 3') and a reverse primer 1492r (5'- TAC GGC TTA CCT TGT TAC GA -3'). PCR master mix was made with a total volume of 50 µL consisting of 2 µL forward primer, 2 µL reverse primer, 2 µL DNA template, 25 µL My Taq HS red DNA polymerase (bioline), and 19 µL ddH2O. The mixture is homogenized and spindown is then put in a thermocycler [28]. PCR products were confirmed using electrophoresis and visualized with UVIDoc HD5.

DNA sequencing by 1st Base through PT. Indonesian Science Genetics. The DNA sequencing process was carried out using the dideoxy sanger method. The DNA sequences obtained were processed using the Basic Local Alignment Search Tool (BLAST) database tracking program on the website of the National Center for Biotechnology Information, National Institute for Health, USA, namely www.ncbi.nlm.nih.gov. Sequences were processed with the Mega11 program. Sequence characterization was performed by aligning.

3 Results and discussion

3.1 Characteristics of *Sinularia* sp. soft coral symbion bacteria

Isolation of symbiont bacteria from soft coral *Sinularia* sp. carried out by serial dilution from 10-1 to 10-8 using sterile seawater as a solvent. Nine bacterial symbionts *Sinularia* sp. successfully isolated with different colony and cell morphology. Each symbiont bacterial isolate is named with the code LA1, LA2, LA3, LA5, LA6, LA7, LA8, LA10, and LA11.

The symbiont bacterial isolates were observed macroscopically (Figure 1) and microscopically (Figure 2). Following are the results of the characterization of the soft coral symbiont bacteria *Sinularia* sp. (Table 1).

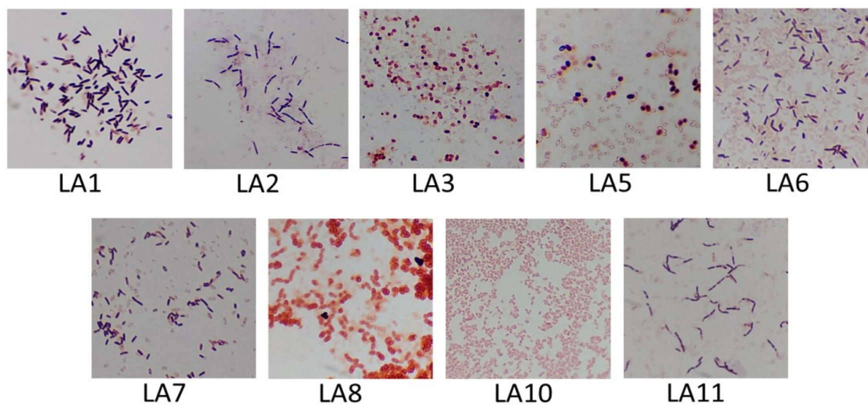


Fig. 1. The results of Gram staining of *Sinularia* sp. soft coral symbiont bacterial.

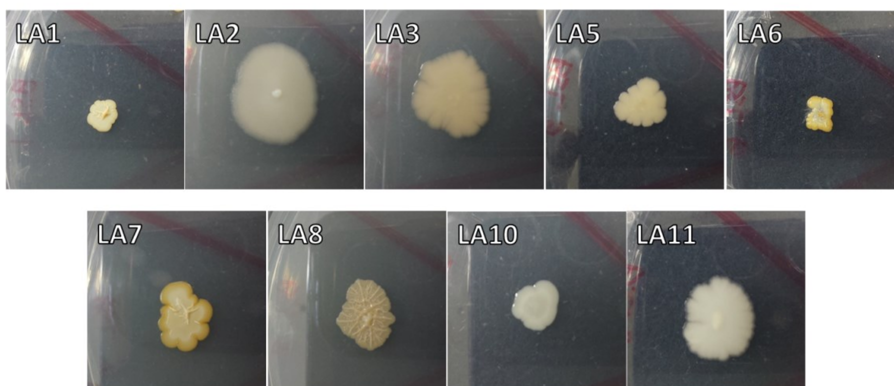


Fig. 2. Results of macroscopic observations of *Sinularia* sp. soft coral symbiont bacterial.

Table 1. Characteristic data of soft coral symbiont bacteria *Sinularia* sp.

Isolate	Shape	Margin	Elevation	Texture	Colony Color	Gram	Cell Shape
LA1	irregular	lobate	umbonate	dry	light yellow	+	rod
LA2	round	irregular	umbonate	mucoid	milk white	+	rod
LA3	irregular	lobate	flat	moist	beige	-	spherical
LA5	irregular	irregular	convex	moist	beige	+	spherical
LA6	irregular	lobate	flat, raised margin	mucoid	yellow	+	rod
LA7	irregular	lobate	umbonate	dry	yellow	+	rod
LA8	irregular	undulate	lobate	dry	beige	-	rod
LA10	round	smooth	convex	moist	milk white	-	rod
LA11	round	irregular	raised	mucoid	white	+	rod

Macroscopic observations by looking at the morphology of the symbiont bacterial colonies and based on the Microbiology Laboratory Theory and Application Leboffe and Pierce guidebook (2012). Microscopic observation of the symbiont bacteria was carried out by looking at the cell morphology and the grouping of Gram-positive or Gram-negative bacteria. Appearance of Gram stain, Gram negative bacterial cells are red and Gram-positive bacterial cells are purple. There are differences in the peptidoglycan wall between Gram positive and negative bacteria. According to Tripathi and Sapra [29], Gram staining is a method used to distinguish between Gram positive and Gram-negative bacteria. Gram positive bacteria will appear purple and Gram-negative bacteria will appear red when observed under a microscope. The initial dye for Gram staining is crystal violet. Iodine material will form a violet-iodine crystal complex to prevent the purple colour from fading easily. The decolourizer process with the addition of ethanol and acetone was carried out to remove the colour from the crystal violet. The principle of Gram staining is based on the ability of the bacterial cell wall to maintain the crystal violet color during treatment. The cell wall of Gram-positive bacteria has a higher peptidoglycan content than Gram negative bacteria which has a high lipid content. Gram positive and Gram-negative bacteria are able to absorb crystal violet color. The lipid layer on the cell wall of Gram-negative bacteria is easily shed so that the crystal violet color will be shed during the decolorization process. The addition of safranin dye to the sample aims to give a red color to Gram negative bacteria.

The results of observations of the symbiont bacteria *Sinularia* sp. which is summarized in the table shows that there are 3 isolates with round shapes and 6 isolates with irregular shapes. Colony margins varied greatly, 4 isolates had lobate margins, 3 isolates had irregular margins, 1 isolate had undulate margins, and 1 isolate had smooth margins. There are also isolates with dry, mucoid, and moist textures. Observations from Gram staining of the symbiont bacteria *Sinularia* sp. showed that there were 3 Gram negative bacteria and 6 Gram positive bacteria. The diversity of bacteria obtained will be continued for antibacterial activity tests against *P. acnes* and *S. epidermidis*.

3.2 Antibacterial activity test

Antibacterials are compounds that can inhibit the growth or kill bacteria. In this study, the antibacterial activity test of the symbiont bacteria *Sinularia* sp. against acne-causing bacteria, namely Propionibacterium acne and Staphylococcus epidermidis. *P. acnes* is a Gram-positive bacterium with bacilli-shaped cells. *S. epidermidis* is a Gram-positive bacterium with a coccal cell shape. The results of Gram staining for *P. acnes* can be seen in Figure 3 and *S. epidermidis* can be seen in Figure 4. Gram staining of the tested bacterial isolates aims to determine the purity of the test bacteria by looking at the uniformity of the test bacterial cells.

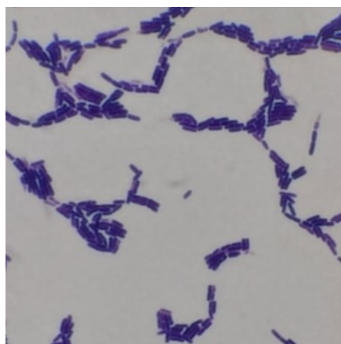


Fig. 3. *Propionibacterium acnes* cell morphology.

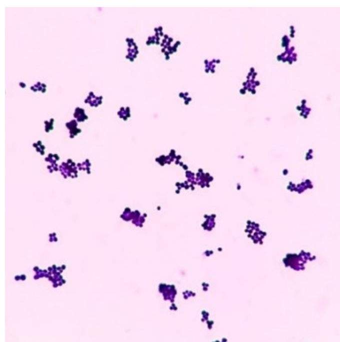


Fig. 4. *Staphylococcus epidermidis* cell morphology.

For the initial stage, an antibacterial screening process will be carried out to obtain isolates of the symbiont bacteria *Sinularia* sp. which can produce antibacterial compounds. The method used is disk diffusion. Bacteria that have antibacterial activity will inhibit the growth of pathogenic bacteria, as evidenced by the clear zone that forms around the disc paper. The results of the screening of the antibacterial activity of the symbiont bacteria *Sinularia* sp. against *P. acnes* and *S. epidermidis* can be seen in Figures 5 and 6**.**

The results of the screening of the antibacterial activity of the symbiont bacteria *Sinularia* sp. against *P. acnes* and *S. epidermidis* showed that there were several isolates that formed inhibition zones. The inhibition zone resulting from the antibacterial test results indicates the activity of inhibiting the growth of the tested bacteria. Isolate with the best inhibition zone was re-tested for its antibacterial activity to categorize the level of its antibacterial activity. strength. Data from the screening results for antibacterial activity are summarized in Table 2.

Table 2. Antibacterial activity screening results.

Isolate Code	Inhibition Zone	
	Propionibacterium acnes	*Staphylococcus epidermidis*
LA1	+	+
LA2	+	-
LA3	-	-
LA5	-	+
LA6	+	+
LA7	+	+
LA8	-	-
LA10	-	-
LA11	-	-

Note:
(+) inhibition zone formed (positive for antibacterial activity)
(-) no inhibition zone is formed (negative has antibacterial activity)

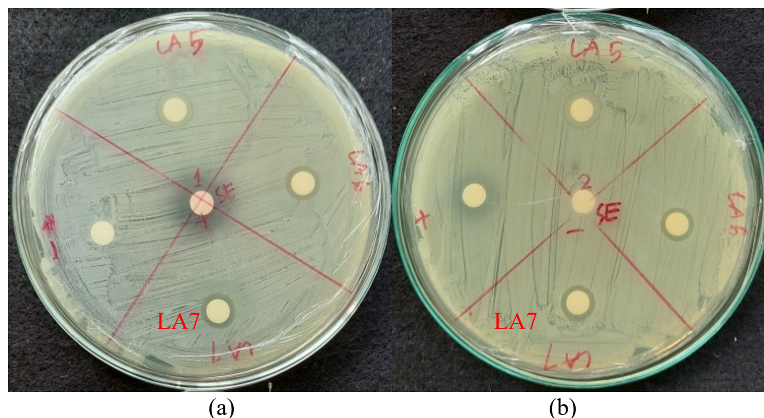


Fig. 5. The results of the screening test for antibacterial activity of the *Sinularia* sp. symbiont bacteria against *P. acnes* (a) replicate 1 (b) replicate 2.

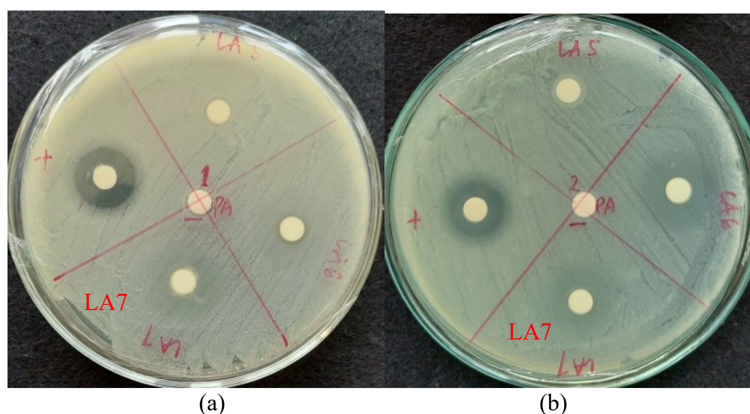
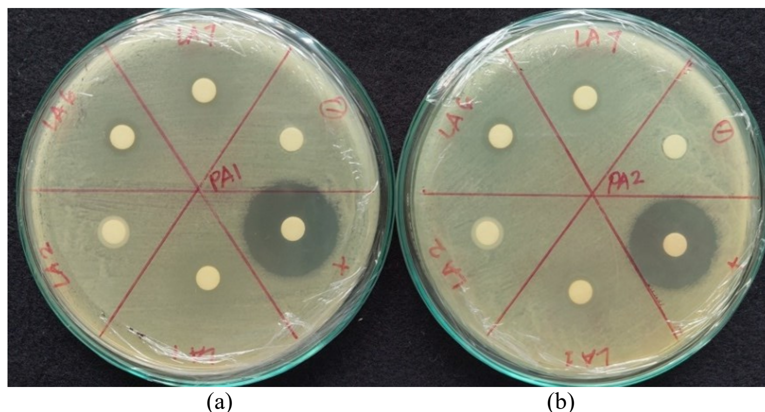


Fig. 6. The results of the screening test for antibacterial activity of the *Sinularia* sp. symbiont bacteria against *S. epidermidis* (a) replicate 1 (b) replicate 2.

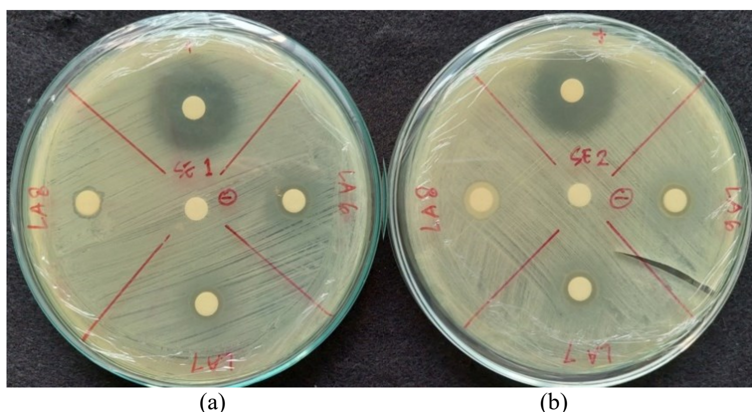
Based on the data in Table 2, it can be seen that the isolates LA1, LA2, LA6, and LA7 have the potential to inhibit the growth of *P. acnes* because they form a clear zone around the paper disk. It can also be seen that the isolates LA6, LA7, and LA11 were able to inhibit the growth of *S. epidermidis* because they formed an inhibition zone around the paper disk. Isolates that have the potential for antibacterial activity will be tested for antibacterial activity to determine the strength of the antibacterial activity so that the level of inhibition can be categorized based on the size of the inhibition zone formed. The inhibition zone formed around the disc paper was measured with a vernier caliper. The results of the antibacterial activity test can be seen in Figures 7 and Figures 8. The results of measuring the diameter of the inhibition zone are summarized in Tables 3 and 4.

Based on the data from the antibacterial activity test results in Tables 3 and 4, it can be seen that several isolates formed an inhibition zone. The largest diameter inhibition zone was formed in LA7 isolate. The size of the inhibition zone formed is influenced by several factors, namely competition between the symbiont bacteria *Sinularia* sp. and pathogenic bacteria as well as the production of secondary metabolites in the form of bioactive compounds. According to Chevrette et. al. [30], secondary metabolites produced by bacteria are the main source of antibiotics and other bioactive compounds. Within the microbial community, these molecules can mediate interspecies interactions and responses to environmental changes.



(a) (b)

Fig. 7. Results of antibacterial activity test of isolates LA1, LA2, LA6, and LA7 against *P. acnes* (a) Replication 1 (b) Replication 2.



(a) (b)

Fig. 8. Antibacterial activity test results of isolates LA1, LA3, LA5, LA6, LA7, LA8, and LA11 against *S. epidermidis* (a) Replication 1 (b) Replication 2.

Table 3. The results of the antibacterial activity test of the symbiont *Sinularia* sp. Isolate code LA1, LA3, LA5, LA6, LA7, LA8, and LA11 against *S. epidermidis*.

Isolate Code	**Inhibition Zone Diameter (mm)**		**Average (mm)**
	Replication 1	Replication 2	
LA1	13,2	15,6	14,4
LA5	6,3	4,6	5,5
LA11	5,1	4,5	4,8
LA6	13,9	14,3	14,1
LA7	14,7	14,4	14,6
Positive control	19,6	17,9	18,75
Negative control	0	0	0

Table 4. The results of the screening of the antibacterial activity of the symbiont bacteria *Sinularia* sp. code isolates LA1, LA2, LA6, and LA7 against *P. acnes*.

Isolate Code	Inhibition Zone Diameter (mm)		Average (mm)
	Replication 1	Replication 2	
LA1	13,6	15,2	14,4
LA2	5,5	5,7	5,6
LA6	15,4	15,5	15,5
LA7	17,6	15,7	16,6
Positive control	19,3	17,7	18,5
Negative control	0	0	0

Based on the antibacterial activity test against *S. epidermidis*, LA7 isolate formed an inhibition zone of 14.6 mm which indicates that the bacteria has moderate category of antibacterial activity. In *P. acnes*, LA7 isolate formed an inhibition zone of 16.6 mm indicating that LA7 isolate has strong antibacterial activity. According to Cita et. al. [31] antibacterial activity based on the size of the inhibition zone formed around the disc paper was classified as strong for inhibition zone diameter (id) > 16.0 mm, medium (good) for diameters ranging from 11 to 16 mm, weak for diameters 7–11 mm and no activity for diameters <7 mm. From the results of the antibacterial activity test, LA7 isolate with the best activity will continue to identify species based on the 16S rRNA gene. LA7 isolate is a Gram positive bacterium with bacilli form.

3.3 Analysis of Bioactive Compounds with GC-MS method

Gas chromatography and mass spectrometry have broad analytical applications that can be used as summaries and mixtures of several components of chemical compounds [32]. The results of the GC-MS crude extract of isolate LA7 are displayed in the form of a chromatogram (Figure 9). After processing the data, there were 81 compounds detected from Isolate LA7 (Table 5).

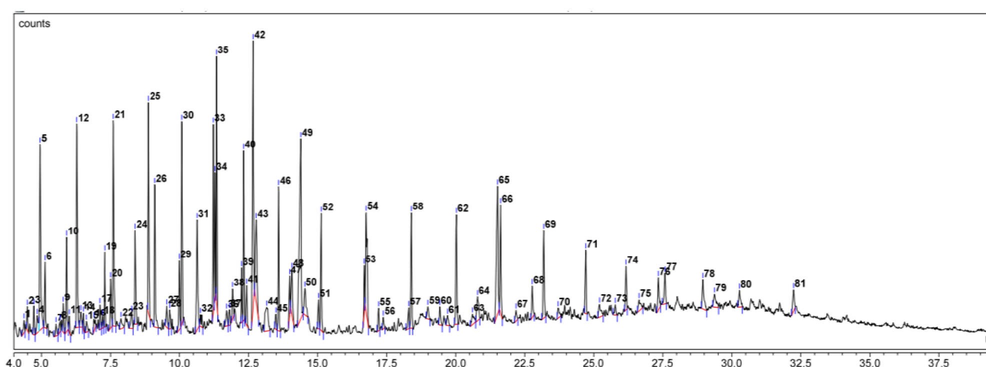


Fig. 9. Chromatogram of GC-MS results of isolate LA7.

Table 5. Compound from GC-MS analysis isolate LA7.

Peak	Ret. Time (min)	Compound	Chemical Formula	Rel. Area (%)
1	4,37	Octadecane, 6-methyl-	C19H40	0,32
2	4,48	1-Octadecanesulphonyl chloride	C18H37ClO2S	0,39
3	4,72	2-Tridecen-1-ol, (E)-	C13H26O	0,46
4	4,84	3-Trifluoroacetoxydodecane	C14H25F3O2	0,52
5	4,95	Dodecane	C12H26	3,94
6	5,13	Undecane, 2,6-dimethyl-	C13H28	1,01
7	5,52	Undec-10-ynoic acid, dodecyl ester	C23H42O2	0,25
8	5,67	2-Piperidinone, N-[4-bromo-n-butyl]-	C9H16BrNO	0,27
9	5,79	Undecane, 4,8-dimethyl-	C13H28	0,45
10	5,91	Octane, 2,3,7-trimethyl-	C11H24	1,52
11	6,01	Tetradecane, 2,6,10-trimethyl-	C17H36	0,24
12	6,28	Tridecane	C13H28	3,54
13	6,43	Bicyclo[4.4.1]undeca-1,3,5,7,9-pentaene	C11H10	0,41
14	6,52	Octadecane, 6-methyl-	C19H40	0,20
15	6,63	Tetradecane, 2,6,10-trimethyl-	C17H36	0,26
16	6,90	9,12,15-Octadecatrienoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl ester, (Z,Z,Z)-	C25H40O6	0,32
17	7,12	Tetradecane, 2,6,10-trimethyl-	C17H36	0,42
18	7,21	Octadecane, 6-methyl-	C19H40	0,19
19	7,29	Dodecane, 2,6,10-trimethyl-	C15H32	0,94
20	7,50	4-Trifluoroacetoxytetradecane	C16H29F3O2	0,93
21	7,60	Tetradecane	C14H30	3,62
22	7,88	2,6,10-trimethylundecanoic Acid, 2,2,2-trifluoroethyl ester	C16H29F3O2	0,39
23	8,27	2-Trifluoroacetoxypentadecane	C17H31F3O2	0,46
24	8,39	Heptadecane, 2,6,10,14-tetramethyl-	C21H44	1,56
25	8,87	Pentadecane	C15H32	3,30
26	9,10	Butylated Hydroxytoluene	C15H24O	2,17
27	9,54	1-Hexadecanol, 2-methyl-	C17H36O	0,45
28	9,64	Tetradecane, 2,6,10-trimethyl-	C17H36	0,42
29	10,00	1-Decanol, 2-hexyl-	C16H34O	1,20
30	10,08	Hexadecane	C16H34	2,88
31	10,64	Tetradecane, 2,6,10-trimethyl-	C17H36	2,75
32	10,76	1-Hexadecanol, 2-methyl-	C17H36O	0,37
33	11,22	Heptadecane	C17H36	2,35
34	11,29	Pentadecane, 2,6,10-trimethyl-	C18H38	1,60
35	11,34	Dodecane, 1-cyclopentyl-4-(3-cyclopentylpropyl)-	C25H48	2,82
36	11,70	1-Hexadecanol, 2-methyl-	C17H36O	0,43
37	11,81	Octadecane, 6-methyl-	C19H40	0,31
38	11,92	Ethanol, 2-(octadecyloxy)-	C20H42O2	0,65
39	12,24	5-Octadecene, (E)-	C18H36	0,74
40	12,32	Eicosane	C20H42	2,21
41	12,43	Tetradecane, 2,6,10-trimethyl-	C17H36	0,69
42	12,66	Bicyclo[3.3.1]nonane-2,8-dione	C9H12O2	4,93
43	12,78	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C11H18N2O2	2,61
44	13,15	1,3,2-Dioxaborinane, 2,4-diethyl-5-methyl-6-propyl-	C11H23BO2	1,53

Peak	Ret. Time (min)	Compound	Chemical Formula	Rel. Area (%)
45	13,49	Tetradecane, 2,6,10-trimethyl-	C17H36	0,33
46	13,59	Eicosane	C20H42	2,31
47	13,98	Hexadecanoic acid, methyl ester	C17H34O2	0,63
48	14,07	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C11H18N2O2	1,23
49	14,39	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C11H18N2O2	6,15
50	14,54	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C11H18N2O2	0,89
51	15,03	1-Decanol, 2-hexyl-	C16H34O	0,52
52	15,13	Eicosane	C20H42	2,08
53	16,69	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C19H34O2	0,82
54	16,76	Eicosane	C20H42	3,07
55	17,21	Heptadecanoic acid, 16-methyl-, methyl ester	C19H38O2	0,43
56	17,38	Tetradecane, 2,6,10-trimethyl-	C17H36	0,26
57	18,30	1-Decanol, 2-hexyl-	C16H34O	0,44
58	18,39	Eicosane	C20H42	2,08
59	18,99	Tetradecane, 2,6,10-trimethyl-	C17H36	0,34
60	19,42	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2	0,43
61	19,70	tert-Hexadecanethiol	C16H34S	0,33
62	20,03	Eicosane	C20H42	2,22
63	20,61	tert-Hexadecanethiol	C16H34S	0,46
64	20,79	Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'a,10a)-	C33H37N5O5	0,93
65	21,51	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C14H16N2O2	4,03
66	21,63	Eicosane	C20H42	2,01
67	22,18	tert-Hexadecanethiol	C16H34S	0,29
68	22,77	Octadecanoic acid, 2-propenyl ester	C21H40O2	0,70
69	23,19	Eicosane	C20H42	1,84
70	23,71	tert-Hexadecanethiol	C16H34S	0,33
71	24,71	Eicosane	C20H42	1,24
72	25,21	tert-Hexadecanethiol	C16H34S	0,55
73	25,77	Tetrapentacontane, 1,54-dibromo-	C54H108Br2	0,27
74	26,17	Tetradecane, 2,6,10-trimethyl-	C17H36	0,90
75	26,65	tert-Hexadecanethiol	C16H34S	0,49
76	27,34	9-Octadecenamide	C18H35NO	0,87
77	27,58	Tetradecane, 2,6,10-trimethyl-	C17H36	0,75
78	28,95	tert-Hexadecanethiol	C16H34S	0,96
79	29,37	tert-Hexadecanethiol	C16H34S	0,61
80	30,27	tert-Hexadecanethiol	C16H34S	0,53
81	32,24	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2	0,66

Based on the GC-MS data, there were identified antibacterial compounds at high concentrations namely Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (6.15%). Several studies related to the compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- have been carried out, it was found that the compound has antibacterial and antioxidant activity [33,34]. The second bioactive compound was Bicyclo[3.3.1]nonane-2,8-dione (4,93%) which potential as anticancer chemotherapeutics

[35]. The third was Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-(4,03%) which has strong antioxidant activity [36,37]. Based on this data, it proved that the isolate LA7 has potential against acne causing bacteria (*P. acnes* and *S. epidermidis*).

3.4 Molecular 16S rRNA identification

Bacteria with isolate code LA7 which has the best antibacterial activity against *P. acnes* and *S. epidermidis* were identified molecularly. Species identification using 16S rRNA gene markers. To obtain DNA isolate LA7, DNA extraction was performed using the InstaGenTM matrix protocol. DNA purity was measured by a spectrophotometer nanodrop. The results of measuring the purity of LA7 isolate can be seen in Table 6.

Table 6. Results of nanodrop spectrophotometry of DNA isolate LA7.

Sample	Concentration (ng/µl)	Wavelength		Ratio $A\lambda260/A\lambda80$
		$\lambda260$	$\lambda280$	
LA7	342,3	6,846	3,252	2,11

The table shows that the purity of the DNA isolate LA7 is 2,11. Extracted DNA can be used as a template in PCR 16S rRNA gene amplification. According to Hindash and Hindash [38], DNA purity can be determined quantitatively with a nanodrop spectrophotometer at an OD ratio of 260/280. Based on the OD 260/280 ratio, DNA is declared pure from protein if it is less than 1.8 and pure from RNA if it is more than 2.0.

The results of DNA isolation from LA7 isolate were amplified by the 16S rRNA gene by PCR. To determine the success of amplification of LA7 isolate, agarose gel electrophoresis was performed. According to Balakrishnan et. al. [39], the amplicon size of the 16S rRNA gene is in the range of 1500 bp which consists of a conserved region. The amplification of the 16S rRNA gene was declared successful if the amplicon visualized by gel electrophoresis showed a size of 1500 bp. Visualization of the electrophoretic gel in Figure 10 shows the amplicon size of isolate LA7, which is 1500 bp, indicating that the 16S rRNA gene was successfully amplified. The results of the amplification of LA7 isolate will then be sequenced to determine the nucleotide base sequence. The results of the 16S rRNA gene amplification can be seen in Figure 10.

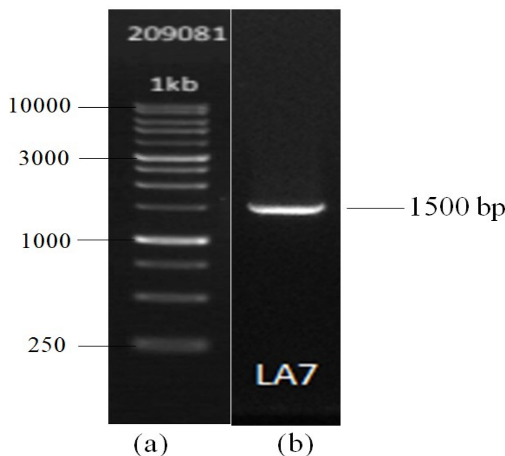


Fig. 10. Electrophoresis of 16S rRNA gene amplification using PCR. (a) DNA Ladder 1kb (b) Amplicon band 16S rRNA Isolate LA7.

The sequencing results of LA7 isolates were analyzed using BLAST (Basic Local Alignment Search Tools) on the NCBI online page. The BLAST analysis aims to compare the LA7 sequence with the DNA sequence data in GenBank. The results of the BLAST analysis are listed in Table 7.

Table 7. Results of BLAST analysis of bacterial isolate LA7.

Name of Bacteria	*Max score*	*Total Score*	*Query Cover*	E-Value	*Per. Ident*
Bacillus aerius	2100	2100	100%	0.0	98.17%
Bacillus stratosphericus	2097	2097	100%	0.0	98.08%
Bacillus altitudinis	2095	2095	100%	0.0	98.08%
Bacillus pumilus	2095	2095	100%	0.0	98.08%
Bacillus aerophilus	2095	2095	100%	0.0	98.08%

The results of the BLAST sequence of isolate LA7 showed the highest homology value, namely 98.17%, which means that LA7 has species-level similarities with Bacillus aerius. Moritania et al. [40] that in the BLAST results, homology values (percent identity) of more than 97% can represent similarities at the species level. The homology (percent identity) value of 93% - 97% can represent identity at the genus level but differs at the species level. Percent identity with a value below 93% means the possibility of a new species whose nitrogen base sequence has not been included in the Genbank database. Based on Table 7, isolate LA7 has the highest similarity with Bacillus aerius with a maximum score of 2100, a total score of 2100, 100% query coverage, 0.0 E-value, and 98.17% identity percentage. According to Claverie and Notredame [41], the maximum score and total score are the number of alignment segments from the database sequence that match the nucleotide sequences. The score value indicates the accuracy of the alignment of the unknown nucleotide sequence with the data in GenBank, the higher the value the higher the homology level of the two sequences. The E-value is an estimated value that provides a statistically significant measure of both sequences. A higher E-value indicates a lower level of homology between sequences, while a low E-value indicates a higher level of homology between sequences. The E-value is 0 (zero) indicating that the query sequence is similar to the sequence in GenBank. Query coverage shows the percentage of database covered by queries. After the LA7 sequences were analyzed with BLAST, phylogenetic tree analysis was performed. Phylogenetic tree analysis using the "Neighbor Joining" approach, and boostrap 1000X. According to Rusinko and McPartlon [42], neighbour joining (NJ) is a taxonomic method that uses distance as a principle for grouping taxa. The grouping of these taxa is based on calculating the evolutionary distance of pairs of operational taxonomic units where each branch of the phylogenetic tree evolves at a different speed. According to Dey et. al. [43], bootstrap values are used to estimate the confidence level of a phylogenetic tree. The higher the bootstrap value, the higher the level of confidence in the reconstructed tree topology. This is due to random effects in the distribution of characters in the data. The phylogenetic tree of isolate LA7 can be seen in Figure 11.

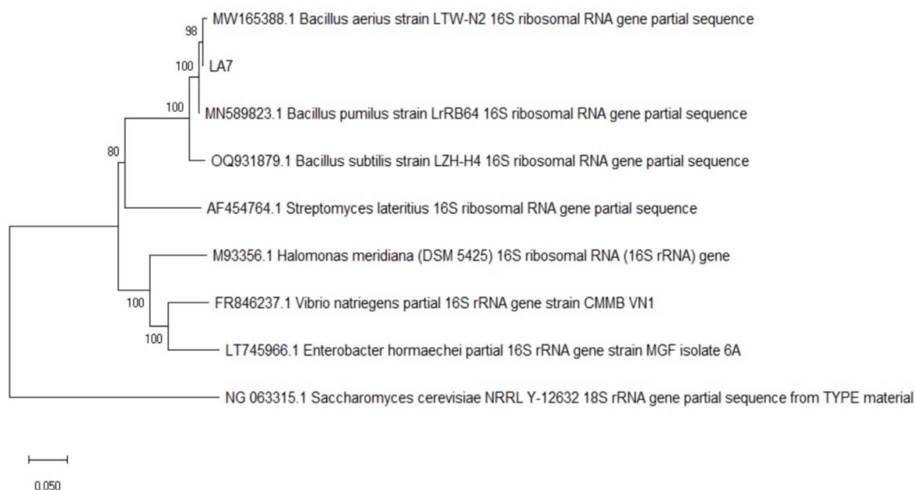


Fig. 11. The phylogenetic tree of isolate LA7 showed it has high similarity with *B. aerius*.

The evolutionary history was inferred using the Neighbor-Joining method [44]. The optimal tree with the sum of branch length = 1.54531298 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [45] and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1760 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [46].

The phylogenetic analysis of LA7 isolates was carried out by comparing the bacterium with several other species, consisting of in group and out group. The in group was selected from the BLAST results, namely *Bacillus aerius* and *Bacillus pumilus*. In the group of the genus Bacillus used is *Bacillus subtilis*. The other in group used was bacteria that had in common with isolate LA7, antibacterial activity against *P. acnes* and *S. epidermidis*. These bacteria are *Streptomyces lateritius*, *Halomonas meridian*, *Enterobacter hormaechei*, and *Vibrio natriengens*. The selection of the out group came from a fungus, namely *Saccharomyces cerevisiae*. Subari et al. [47] stated that the purpose of using outgroups in constructing phylogenetic trees is to identify primitive (plesiomorphic) and derived (apomorphic) characteristics of ingroup groups, as well as to establish a starting point in constructing a phylogenetic tree.

Based on the cladogram in Figure 11, isolate LA7 has a close relationship with *Bacillus aerius* with a bootstrap value of 98. This bootstrap value has a high level of confidence. According to Subari et al. [47] the bootstrap value is the value used to test how well the model data set is used, if the bootstrap value is low then the sequence of the analysis to obtain a phylogenetic tree cannot be trusted. A phylogenetic tree that has a high and good level of confidence is a phylogenetic tree with a bootstrap value above 70.

Bacillus aerius is a gram-positive bacillus in the form of bacilli. Bacillus aerius bacteria are halotolerant, able to live in places with a salt concentration of 0-30% [48]. *Bacillus aerius* is known as a gastrointestinal microbe because it is found in the digestive tract of catfish [49], sea sponges [50], and grouper fish [51], the genus Bacillus is also widely known as a probiotic in fish. Gray [52] found that *Bacillus aerius* can inhibit the replication of Methicillin-Resistant Staphylococcus aureus (MRSA). MRSA is known as a human skin pathogen which

is very difficult to cure because of its resistance to the antibiotic methicillin. The study conducted by Galaviz-Silva et al. [53] reported antimicrobial activity against *Staphylococcus aureus* by *Bacillus aerius* isolated from marine habitats in Mexico. The results of this study indicate that this marine bacterium has potential as an alternative in the development of new antimicrobials against other clinically important bacteria. According to Saun et al. [54]; Saun, Mehta and Gupta [55], *Bacillus aerius* has not been reported to have directly antagonistic activity, but several reports revealed that *Bacillus aerius* produces a thermophilic lipase with antitumor properties, making it a possible candidate for therapeutic applications.

Based on several studies, *Bacillus aerius* has potential as a bacterium that can be developed in the medical field to fight pathogenic bacteria that cause disease in humans. The halotolerant nature of B. aerius facilitates the process of making pharmaceutical products under high salinity conditions.

4 Conclusions

Soft coral symbiont bacteria *Sinularia* sp. with isolates code LA1, LA2, LA6, LA7 could inhibit the growth of *P. acnes* and isolates LA1, LA3, LA5, LA6, LA7, LA11 could inhibit the growth of *S. epidermidis.* LA7 isolate had the best antibacterial activity with strong category of antibacterial activity against *P. acnes* and medium category of antibacterial activity against *S. epidermidis*. The results of species identification based on the 16S rRNA gene showed that LA7 isolate was *Bacillus aerius* with a homology value of 98.17%. The result of GCMS, indicated that *Bacillus aerius* could produce Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) ($C_{11}H_{18}N_2O_2$), Bicyclo[3.3.1]nonane-2,8-dione ($C_9H_{12}O_2$), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- ($C_{14}H_{16}N_2O_2$) which potential as anti-bacterial, anti-cancer and antioxidant activity.

For the future work, it is important to do the Whole Genome Sequencing (WGS) of *B. aerius* to get the whole data of sequence and detect the potential gene cluster which encode those three potential compounds as anti-bacterial, anti-cancer and antioxidant activity. Furthermore, it is possible to do the cloning of the genes from three potential compounds and to determine the biosynthetic pathway. Then to apply the downstream processing of the product which could be used in the Industrial system.

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