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Soil bacteria from Muna Island, Southeast Sulawesi, Indonesia: Antibacterial and antibiofilm activities, and the presence of antibiotic-biosynthetic genes

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ABSTRACT

Unexplored soil bacteria isolated from Muna Island, Southeast Sulawesi, Indonesia, provide a new source of active molecules serving as prospective antibacterial agents. In the present research, the antibacterial and antibiofilm activities of bacterial isolates obtained from Muna Island were evaluated. Based on the preliminary screening through the antagonism assay, 5 out of the 15 isolates exhibited various spectra of antibacterial activities. At a concentration of 80 mg/ml, metabolites extracted from the two selected isolates, M7 and P1 inhibited the growth of the five multidrug-resistant strains. These extracts exhibited minimum inhibitory concentration and minimum bactericidal concentration values of 312.5 µg/ml to more than 10,000 µg/ml. An analysis of the 16S rRNA sequences revealed that the corresponding isolates were closely related to *Bacillus aerius* strain 24K and *Priestia* (*Bacillus*) *aryabhattai* strain B8W22, with >97% similarity. Some genes involved in antibiotic biosynthesis, such as *mlnA*, *baeR*, *srfA*, *dfnD*, and *bacD*, were also present in the bacterial genome. Liquid chromatography-tandem mass spectrometry analysis of the most potent M7 extract showed that it contained several antibacterial compounds, such as cyclo(D-Pro-L-Tyr), marinoquinoline G, and rancinamycin Ib.

INTRODUCTION

Infectious diseases are major health concerns worldwide. These infections are commonly treated with a single antibiotic or a combination of some antibiotics [1]. However, improper, and excessive use of antibiotics may cause bacteria to develop various resistance mechanisms, hindering the treatment of patients with infections caused by resistant strains and possibly promoting the risk of mortality [2]. Currently, antibiotic resistance-related diseases are annually responsible for 700,000 deaths worldwide [3]. This number is predicted to increase in the coming years if more effective antibiotics are not discovered. Therefore, further research into new sources of antibiotics is required to overcome this problem.

Soil provides a favorable habitat for numerous microorganisms, including bacteria, fungi, viruses, and protozoa. Generally, 1 g of soil contains bacteria at up to 10^6–10^8 colony-forming units (CFU)/g [4]. Therefore, this environment is a potential source for isolating bacteria, particularly those capable of producing antibiotics. Notably, some new antibiotics, such as surfactin [5], bicyclomycin [6], bacitracin [7], and lysocin E [8] are reportedly produced by soil bacteria. Bacteria antibiotics have received significant attention from the scientific communities because of their structural and functional variations. Most bacterial antibiotics are synthesized through the polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) pathways. NRPS and PKS are multimodular enzymes

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that are commonly involved in the biosynthesis of polyketides and nonribosomal peptides. The NRPS and PKS modules comprise multiple domains that regulate the condensation and elongation of peptides or polyketide polymer chains [9]. For example, bioactive compounds, such as fengycin, iturin, and surfactin, are synthesized through the NRPS pathway, whereas macrolactin, difficidin, and bacillaene are synthesized through the PKS pathway [10]. The detection of genes involved in the production of active microbial compounds may provide valuable insights into the ability of bacteria to produce specific types of metabolites and their potential medicinal applications [11].

Muna Island is located in Central Buton Regency, Southeast Sulawesi, Indonesia. The island has a natural karst hills landscape, which is further distinguished by its dry soils and calcareous rock [12]. Such environmental characteristics allow microbial colonization that may lead to the synthesis of unique antibacterial compounds. Arid environments are the habitats for numerous novel metabolite-producing bacteria [13]. To the best of our knowledge, no research has yet been reported on the diversity and bioprospecting of bacteria in the Soil of Muna Island. Therefore, the present study aimed to analyze the antibacterial and antibiofilm activities of the soil bacteria originating from Muna Island against multidrug-resistant (MDR) strains, along with the molecular identification and detection of microbial active compound-related genes of the most promising isolate.

MATERIALS AND METHODS

Soil collection and MDR strains

A soil sample was obtained from Muna Island, Southeast Sulawesi, Indonesia (GPS location: latitude 4°54'48.8"S and longitude 122°39'56.7"E). The tested isolates of MDR strains, namely *Klebsiella pneumoniae* strain M19, *Pseudomonas aeruginosa* strain M19, *Escherichia coli* strain M4, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Bacillus subtilis* strain M18 were acquired from Dr. Kariadi Central General Hospital (provided by Dr. Rhesi Kristiana, MERO Foundation, Indonesia).

Isolation of soil bacteria

Soil bacteria were isolated using the serial dilution method [14]. In brief, 1 g of the soil sample was placed in 9 ml of sodium chloride solution (0.85%) at 10^{-1} dilution and serially diluting to 10^{-5}. Subsequently, approximately 100 µl of each suspension diluted from 10^{-3} to 10^{-5} was platted on the nutrient agar (NA) medium and incubated at ±28°C for 48 hours. Purified bacterial colonies were further used for preliminary antibacterial screening.

Antagonism assay

Primary screening for the antibacterial activity of soil bacterial isolates toward five MDR strains was performed using a dual culture assay [15]. Each target bacteria was previously cultured on nutrient broth (NB) media for 24 hours at 37°C (OD_{600} = 0.6) and then 1% was inoculated into molten NA and poured into sterile Petri dishes. After the medium solidified, colonies of soil bacteria were streaked around on it, and the Petri dishes were incubated at 37°C overnight. Antibacterial activity was indicated by the formation of an inhibition zone around the colonies. The diameter of the inhibition zone was measured and presented in millimeters. The test was performed in triplicate.

Metabolite extraction

Metabolites from two of the most potent soil bacteria with the broadest antibacterial spectra were extracted to obtain the crude extracts. The isolates were precultured overnight on NB media, and 1% (v/v) of the preculture was inoculated into fresh NB media. Following this, the culture was incubated and shaken at 120 rpm at 28°C overnight. Ethyl acetate was added equally to 2 l of the culture and then shaken for 1 hour at 180 rpm. Subsequently, the upper layer was then separated and evaporated at 50°C [16]. The percentage yield of the crude extract obtained was determined using the following formula:

$$\text{Yield (\%)} = \frac{\text{Extract weight (g)}}{\text{Culture volume (ml)}} \times 100.$$

Antibacterial test of bacterial crude extract (disc-diffusion assay)

The antibacterial activity of metabolite extracts from two potential isolates was evaluated by dripping 20 µl (80 mg/ml) of the extract dissolved in dimethyl sulfoxide (DMSO) on a sterile paper disc, then placing it on Mueller Hinton Agar (MHA) medium that had been inoculated with 1% (v/v) target strains. DMSO and tetracycline (200 µg/ml) were used as negative and positive controls, respectively. The diameters of the inhibitory zones formed after 24 hours of incubation are expressed in millimeters. The test was performed in triplicate [17].

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

MIC and MBC of each extract were determined using a microbroth-dilution assay [18]. The turbidity of the target suspension in the NaCl solution was adjusted to a McFarland standard of 0.5, which is equivalent to 1 × 10^8 CFU/ml. Subsequently, two-fold dilutions of extracts were prepared at concentrations of 0.07–10 mg/ml, then added to a microtitre plate containing Mueller Hinton broth (MHB) medium to a final volume of 200 µl/well. The plate was incubated in a shaker at 150 rpm at 37°C for 24 hours. MIC is defined as the lowest extract concentration required to inhibit bacterial growth (clear visible medium). The MBCs were determined by plating 100 µl of the treated culture on the MHA plates. The spread suspension was transferred from the MIC well to the well containing the highest concentration of extract. MBC was defined as the concentration of an extract that killed all the bacteria, as indicated by the absence of bacterial growth on the MHA plates.

Antibiofilm assessment

Briefly, extracts at concentrations of ¼ × MIC, ½ × MIC, 1 × MIC, and 2 × MIC were placed in sterile microtitre 96-well plates with brain heart infusion (BHI) medium.

Furthermore, bacterial targets at 0.5 McFarland standard, which is equivalent to 1 × 10^8 CFU/ml, were inoculated in each well following the incubation period at 37°C with shaking at 120 rpm overnight. Thereafter, the BHI medium was discarded, and the cells were washed two times with NaCl solution (0.85%). Crystal violet (0.1%) was then added to each well followed by 30 minutes of incubation at 37°C. The crystal violet was then removed, and the stained biofilm was washed with 99% DMSO before calculating its absorbance using an enzyme-linked immunosorbent assay reader (ThermoFischer) at a wavelength of 595 nm [18].

Molecular identification of the potential isolates

The potential isolates were identified based on their 16S rRNA sequences. Bacterial genomic DNA was extracted using a DNA extraction kit (Geneproof), following the kit instructions. Amplification of the 16S rRNA sequences was performed using the 1387R (5'-GGGCGGWGTGTACAAGGC-3') and 63F (5'-CAGGCCTAACACATGCAAGTC-3') primers with amplified products of approximately 1,300 bp in size [19]. A total of 50 µl of polymerase chain reaction (PCR) mixture was prepared by mixing 13 µl nuclease-free water, 5 µl 1387R primer (10 µM), 5 µl 63F primer (10 µM), 2 µl genomic DNA (100 ng/µl), and 25 µl 2× GoTaq Green® Master Mix (Promega). The PCR cycling conditions were as follows: predenaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 45 seconds), extension (72°C for 1 minute 45 seconds), and final extension (72°C for 10 minutes) [20]. Electrophoresis of the PCR products was performed on a 1.5% agarose gel at a voltage of 50 V, with 1 × TAE buffer for 50 minutes. DNA was visualized using fluorosafe dyes and observed using a UV transilluminator. The PCR products were sequenced, and the chromatogram quality of the 16S rRNA sequences was analyzed using seqtrace 0.9.0 software. The sequences were aligned using the Nucleotide BLAST, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences were also deposited in the NCBI database. A phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis software version 11.0, using the neighbor-joining tree method and 1,000 bootstrap replications.

Detection of antibiotic-biosynthetic genes

Ten genes involved in different antibiotic-biosynthetic pathways, namely *NRPS*, polyketide synthase type 1 (*PKS 1*) *mlnA*, *dhBE*, *bacD*, *dfnD*, *srfA*, *ituA*, *fenA*, and *baeR* were amplified using specific primers for PCR screening (Table 1). A total of 50 µl PCR mixture was prepared by mixing 2 µl bacterial genomic DNA (100 ng/µl), 5 µl reverse primer (10 µM), 5 µl forward primer (10 µM), 25 µl 2× GoTaq Green® Master Mix (Promega), and adjusted with 13 µl nuclease-free

Table 1. Specific primers, PCR conditions, and amplicon sizes used in this study.

No.	Gene target	Primer	Sequences (5'–3')	Target amplicon size (bp)	PCR conditions	References
1	NRPS	A3F	GCSTACSYSATSTACACSTCSGG	700–800	Predenaturation 95°C 1 minute; 35 cycles of denaturation 94°C 40 seconds, annealing 59°C 1 minute, extension 72°C 1 minute 30 seconds; and final extension 72°C 10 minutes.	[21]
		A7R	SASGTCVCCSGTSCGGTAS			
2	PKS 1	KS2F	GCSATGGAYCCSCARCARCGSVT	700	Predenaturation 94°C 5 minutes; 35 cycles of denaturation 94°C 1 minute, annealing 58°C 1 minute, extension 72°C 1 minute; and final extension 72°C 10 minutes.	[22]
		KSR5	GTSCCSGTSCCRTGSSCYTCSAC			
3	*mlnA*	mlnA-F	GGCAGGGTCATACCTCATAATC	920	Predenaturation 94°C 3 minutes; 35 cycles of denaturation 94°C 45 seconds, annealing 55°C 30 seconds, extension 72°C 1 minute 30 seconds; and final extension 72°C 7 minutes.	[23]
		mlnA-R	AGCAGACTTTCGGTCTCATTC			
4	*fenA*	fenA-F	CATTCATCCTGGAGACCCTATTC	960		
		fenA-R	TAAGACCGCAGGCATGTTATAG			
5	*srfA*	srfA-F	GCTGATGATGAGGAGAGCTATG	890		
		srfA-R	GATGGTCGATACGTCCGATAAA			
6	*ituA*	ituA-F	CGGGAAACAACAGGCAAATC	980		
		ituA-R	CGTCACCAGCGGTGTAAATA			
7	*baeR*	baeR-F	AGACTCCACCAAGGCAAATC	990		
		baeR-R	CAGCGGCTTCATGTCATACT			
8	*dhbE*	dhbE-F	GCTGGAGGAAGAGTGGTATTATC	940	Predenaturation 94°C 3 minutes; 35 cycles of denaturation 94°C 45 seconds, annealing 54°C 30 seconds, extension 72°C 1 minute 30 seconds; and final extension 72°C 7 minutes.	
		dhbE-R	CAGTAAATGAAGCGGCGTTATG			
9	*bacD*	bacD-F	CCGGCGTCAAGTCTATCAAA	670		
		bacD-R	CATGGCTCCTGCTCCAATAA			
10	*dfnD*	dfnD-F	CAGGCGGAATAGGAGAAGTATG	900		
		dfnD-R	CGGCAGCCGATTGAAATAAC			

water. The amplified DNA fragments were visualized on a 1.5% agarose gel and observed using a UV transilluminator.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The most potent extract (M7) was used for analyzing its compound profiling using LC-MS/MS analysis (Xevo G2- XS quadripole time-of-flight mass spectrometer, Waters, USA) with an electron spray interface (ESI). LC-MS/MS was performed as previously described [20]. Each peak was identified using ESI in positive ion mode. The identified mass was analyzed using the library available in the UNIFI software and the website of Natural Products Atlas (https://www.npatlas.org/).

Statistical analysis

Data obtained through the antagonism test, disc diffusion assay, MIC and MBC determination, and antibiofilm assessment, were analyzed and reflected as the average ± standard deviation from three replicates. Statistical significance was determined using one-way analysis of variance continued by Tukey's tests. Statistical significance was set at $p < 0.05$.

RESULTS

Bacterial isolates from Muna Island soil

Soil collected from the Muna Islands contained bacteria at approximately 1.5×10^6 CFU/g. Growing bacterial colonies were then characterized based on their colony morphology (form, color, texture, elevation, size, and margin). Based on their distinctive colony morphologies, 15 isolates were successfully been purified. These isolates were then used for further analyses.

Antibacterial activity of soil bacteria

Fifteen bacterial isolates displayed diverse antibacterial capabilities against five MDR strains (Table 2). Five of the 15 isolates, namely M1, M7, P1, P5, and P6, exhibited antibacterial activity against at least one MDR strain, as indicated by the formation of an inhibitory zone around the endophytic colony (Fig. 1) with inhibitory zone diameters of 4.1 ± 3.5 to 9.3 ± 0.3 mm. The M7 and P1 isolates had the broadest spectra of antibacterial activity because of their ability to inhibit the growth of all MDR strains growth. Therefore, these two isolates were then selected for further analysis.

Extraction yield

The crude extracts of the M7 and P1 isolates had yields of 0.011% and 0.013%, respectively (Table 3). The crude extracts have comparable percentage yields.

Antibacterial activity of crude extract derived from the most potent isolates

At a concentration of 80 mg/ml, the crude extracts of the M7 and P1 isolates inhibited the growth of the five MDR strains. Both extracts exhibited diverse antibacterial activities against the tested strains, as indicated by the range of inhibitory zone sizes (7–8.7 mm). Tetracycline, used as a positive control, also exhibited an inhibitory zone with a diameter of 8–18 mm in all MDR strains. In contrast, DMSO, which was used as a negative control, exhibited no inhibition zones (Table 4).

MIC and MBC of M7 and P1 crude extracts against MDR strains

MIC for M7 and P1 crude extracts against all MDR strains ranged from 312.5 to >10,000 µg/ml (Table 5). Crude

Table 2. Antibacterial activity of soil bacteria against MDR strains tested using the dual culture assay.

No.	Isolate	Diameter of inhibition zone (mm)[a]				
		E. coli strain M4	*K. pneumoniae* strain M19	*P. aeruginosa* strain M19	*B. subtilis* strain M18	MRSA
1	M1	-	-	5.5 ± 1.3	4.5 ± 0.5	6.1 ± 1.5
2	M2	-	-	-	-	-
3	M3	-	-	-	-	-
4	M4	-	-	-	-	-
5	M5	-	-	-	-	-
6	M6	-	-	-	-	-
7	M7	7.8 ± 0.3	9.3 ± 0.3	5.8 ± 0.8	9 ± 1.7	4.1 ± 3.5
8	M8	-	-	-	-	-
9	P1	6.5 ± 0.5	6.7 ± 0.6	4.8 ± 0.1	4.6 ± 0.5	6.1 ± 0.1
10	P2	-	-	-	-	-
11	P3	-	-	-	-	-
12	P4	-	-	-	-	-
13	P5	-	-	-	5.7 ± 0.6	-
14	P6	5.7 ± 5.1	-	-	6.3 ± 0.6	-
15	P7	-	-	-	-	-

[a]Inhibition zones were not formed.

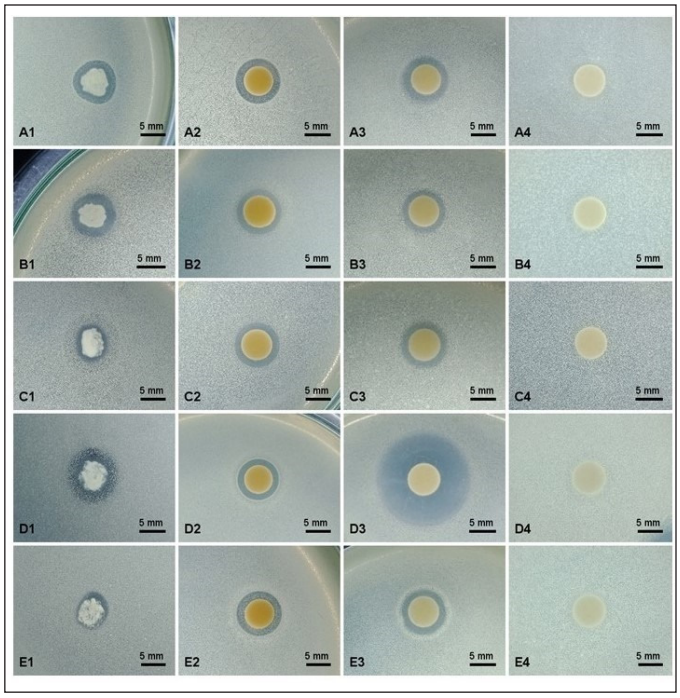

Figure 1. Antibacterial activity of the M7 isolate against *E. coli* strain M4 (A), *K. pneumoniae* strain M19 (B), *P. aeruginosa* strain M19 (C), *B. subtilis* strain M19 (D), and MRSA (E); tested with colony (1), crude extract (80 mg/ml) (2) tetracycline (200 µg/ml) as positive control (3), and DMSO 99% as negative control (4). Scale bar, 5 mm.

Table 3. Yield percentages of crude extracts derived from the most potent isolates.

Crude extract	Culture volume (ml)	Weight (g)	Yield (%)
M7	2,000	0.2257	0.011
P1	2,000	0.2794	0.013

extracts of M7 isolate had the lowest MIC of 312.5 µg/ml against the *E. coli* strain M4 and MRSA, whereas crude extracts of P1 were most effective against the *B. subtilis* strain M18 and *E. coli* strain M4. Lower MIC indicates stronger antibacterial activity. In addition, the MBC of both crude extracts was higher than the MIC for all MDR strains. However, both crude extracts were less active against *K. pneumoniae* strain M19, as shown by the higher MICs and MBCs of all samples compared to those associated with the other strains. This may be because this strain is extremely resistant to the tested concentrations of the crude extracts tested.

Antibiofilm potential

The results of the antibiofilm ability from two selected extracts against all MDR bacterial strains demonstrated that both extracts significantly ($p < 0.05$) reduced MDR biofilm formation in a concentration-dependent manner. The highest inhibition values were recorded for the M7 and P1 extracts at a concentration of 2 × MIC against MRSA and *B. subtilis* strain M18, with inhibition values of 65.1% and 67.25%, respectively. In contrast, the lowest reduction in biofilm formation was recorded for the P1 extract at a concentration of ¼ × MIC, with inhibition values of 5.18% against MRSA (Fig. 2A and B).

Molecular identity of selected isolates

The 16S rRNA analysis showed that the most potent isolates, namely P1 and M7 isolates, shared similarities (>97.5%) with *Bacillus aerius* strain 24K and *Priestia* (*Bacillus*) *aryabhattai* strain B8W22, respectively (Table 6). The 16S rRNA sequences of both isolates could be accessed on the NCBI GenBank database through accession numbers OR066161.1 and OR066162.1. The phylogenetic tree consistently shows that these isolates were closely related to each of their closest related species (Fig. 3).

Table 4. Antibacterial activity of crude extracts of the most potent isolates against MDR strains using the disc-diffusion method.

No	Sample	Diameter of inhibition zone (mm)*				
		E. coli strain M4	*K. pneumoniae* strain M19	*P. aeruginosa* strain M19	*B. subtilis* strain M18	MRSA
1	M7	8.5 ± 0.9^{b}	8.7 ± 1.3^{bc}	8.0 ± 1.0^{bc}	8.6 ± 0.8^{c}	8.0 ± 1.0^{b}
2	P1	8.7 ± 1.0^{b}	7.7 ± 0.3^{b}	7.3 ± 0.6^{b}	7.0 ± 0^{b}	8.7 ± 1.0^{bc}
3	Tetracycline	8.3 ± 0.6^{b}	8.2 ± 0.3^{c}	8.0 ± 0^{c}	18.0 ± 0^{d}	8.8 ± 0.3^{c}
4	DMSO	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}

*Concentrations of crude extracts, tetracycline, and DMSO were 80 mg/ml, 200 µg/ml, and 99%, respectively. Different letters above the number in the same column indicate that the data were statistically significant.

Table 5. MICs and MBCs of M7 and P1 extracts against MDR strains.

Sample	MIC and MBC (µg/ml)									
	E. coli strain M4		*K. pneumoniae* strain M19		*P. aeruginosa* strain M19		*B. subtilis* strain M18		MRSA	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
M7	312.5	>625	5,000	>10,000	1,250	>2,500	625	>1,250	312.5	>625
P1	312.5	>625	10,000	>10,000	625	>1,250	312.5	>625	625	>1,250
Tetracycline	3.91	>7.81	31.25	>62.5	3.91	>7.81	3.91	>7.81	3.91	>7.81

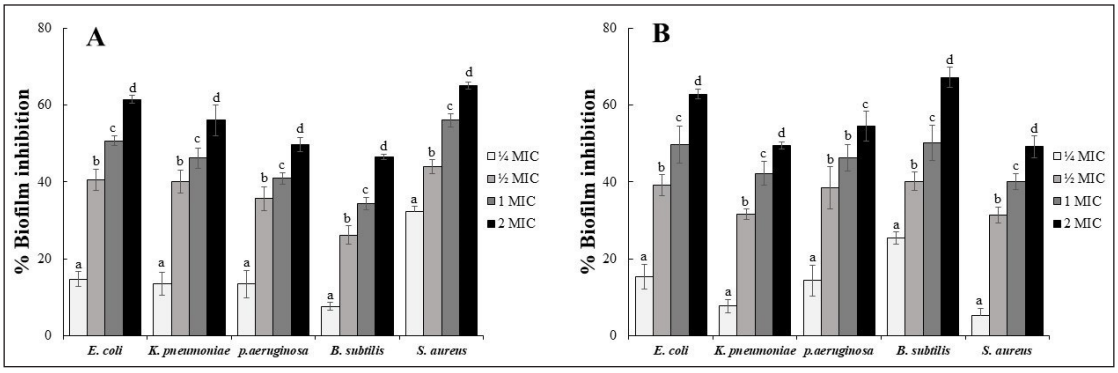

Figure 2. Effect of selected bacterial crude extracts on the biofilm inhibition against MDR clinical isolates cells biofilm. Inhibition activity of crude extract from M7 (A), and P1 (B) isolates.

Table 6. Molecular identity of the selected isolates based on 16S rRNA sequences.

Isolates	Closest relative species (Accession number)	Max score/ total score	E-value	Identity/ query cover (%)	Accession number
M7	*P.* (*Bacillus*) *aryabhattai* strain B8W22 (OR066161.1)	2,241/2,241	0.0	97.65/100	NR 115953.1
P1	*B. aerius* strain 24K (OR066162.1)	1,779/1,779	0.0	99.69/100	NR 118439.1

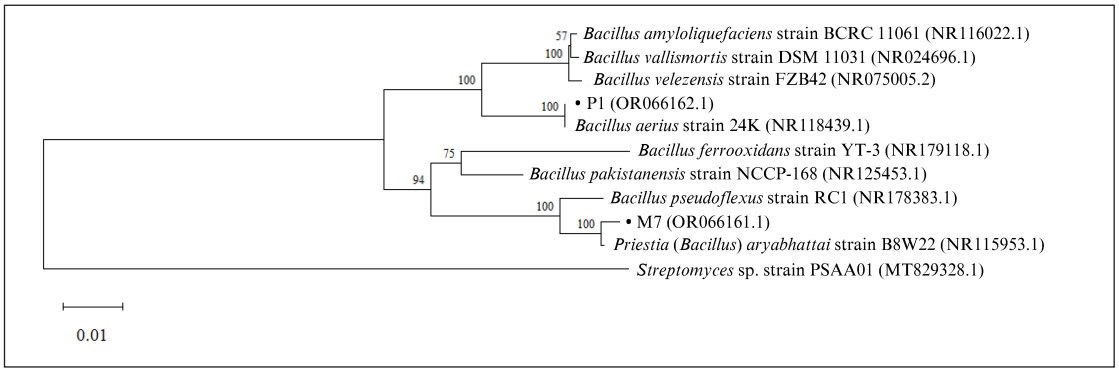

Figure 3. Genetic relationship between M7 and P1 isolates and other *Bacillus*-related strains based on 16S rRNA sequences.

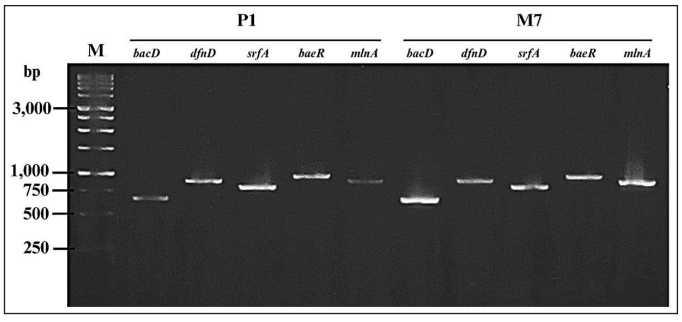

Figure 4. Bands of secondary metabolite biosynthetic genes of P1, and M7 isolates. M represents 1 kb ladder markers on the 1.5% agarose gel.

The presence of metabolite biosynthetic genes

The M7 and P1 isolates were determined to have the *mlnA* (macrolactin biosynthesis), *baeR* (bacillaene biosynthesis), *dfnD* (difficidin biosynthesis), *srfA* (surfactin biosynthesis), and *bacD* (bacilysin biosynthesis) genes. Amplification of these genes revealed the formation of an electrophoretic band of approximately 920 bp for *mlnA*, 900 bp for *dfnD*, 890 bp for *srfA*, 990 bp for *baeR*, and 670 bp for *bacD* (Fig. 4). However, *NRPS*, *PKS* type 1, *dhbE*, *ituA*, and *fenA* were absent in both isolates (Table 7).

Metabolite profile of M7 extract

Because the M7 extract exhibited the most potent antibacterial and antibiofilm activities based on the initial screening, such as disc diffusion, determination of MIC and MBC, and antibiofilm assay, the M7 extract was selected for analyzing its metabolites profile using LC-MS/MS (Supplementary material 1–13). The results revealed that 13 recognized putative compounds were dominant in the extract (Fig. 5). Interestingly, six antibacterial compounds were detected in the extract, including cyclo(D-Pro-L-Tyr), marinoquinoline G, N-carbamoyl-2-hydroxy-3-methoxybenzamide, rancinamycin

Table 7. Presence of antibiotic-biosynthetic genes in two selected isolates.

No.	Target genes*	Isolates	
		M7	P1
1	*NRPS*	-	-
2	*PKS 1*	-	-
3	*mlnA*	+	+
4	*dhbE*	-	-
5	*bacD*	+	+
6	*dfnD*	+	+
7	*srfA*	+	+
8	*ituA*	-	-
9	*fenA*	-	-
10	*baeR*	+	+

*Note: Nonribosomal peptide synthetase, *NRPS*; polyketide synthase type 1, *PKS 1*; *mlnA*, macrolactin biosynthesis; *baeR*, bacillaene biosynthesis; *dfnD*, difficidin biosynthesis; *srfA*, surfactin biosynthesis; and *bacD*, bacilysin biosynthesis

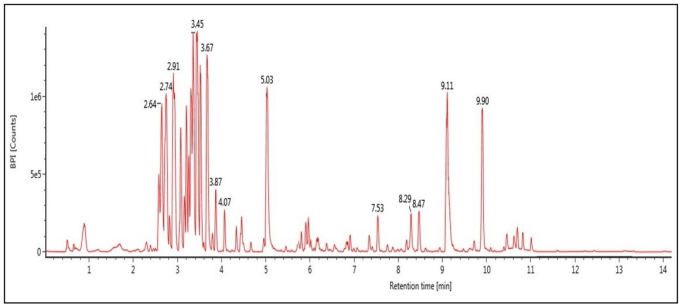

Figure 5. LC-MS/MS chromatogram profile of M7 extract.

Ib, dipyrimicin A, and methyl 6-carbamoylphenazine-1-carboxylate (Table 8).

DISCUSSION

Soil bacteria could serve as sources of diverse antibacterial compounds that play a significant role in the development of antibiotics. In the present study, 15 soil bacterial isolates from Muna Island, Southeast Sulawesi, were screened for their antibacterial activity against five MDR strains, including MRSA. *Bacillus subtilis* strain M18, *P. aeruginosa* strain M19, *E. coli* strain M4, and *K. pneumoniae* strain M19. Five out of the 15 isolates (33%) displayed antagonistic activity to the tested MDR strains. This antagonistic potential is supported by the ability of soil bacteria to produce metabolites that can inhibit the MDR strain growth through numerous mechanisms, including the inhibition of cell wall synthesis, disruption of cell membrane integrity, inhibition of nucleic acid and protein synthesis, and inhibition of bacterial metabolic processes [35]. M7 and P1 were the two most potent isolates because they inhibited the growth of all tested MDR strains. The isolates were selected for metabolite extraction to confirm their antibacterial activity. Notably, the two isolates exhibited similar yields after extraction. Similar conditions in terms of culture time, ethyl acetate solvent-to-bacterial culture ratio, and extraction time were likely responsible for the relatively comparable yields.

Furthermore, the M7 and P1 crude extracts at a concentration of 80 mg/ml showed varied antibacterial activities against the MDR strains, as assessed using the disc-diffusion assay. The effectiveness of antibacterial agents is commonly categorized based on their inhibitory zone diameters, such as weak (<5 mm), moderate (5–10 mm), strong (10–20 mm), and very strong (>20 mm) [36]. Based on this categorization, the M7

Table 8. Metabolite profile of M7 extract.

Retention time (min)	$[M-H]^-$ (*m/z*)	Compounds	Elemental compositions	Other sources	Bioactivities	References
2.62	260.1159	Cyclo(D-Pro-L-Tyr)	$C_{14}H_{16}N_2O_3$	*Streptomyces sp.* strain 22-4	Antibacterial	[24]
2.72	196.1171	Marinoquinoline G	$C_{13}H_{12}N_2$	*Mooreia alkaloidigena* strain CNX-216	Antibacterial	[25]
2.91	226.1295	2,6-Dimethoxy terephthalic acid	$C_{10}H_{10}O_6$	*Streptomyces sp.* strain YIM66017	Antioxidant	[26]
3.45	210.1345	N-Carbamoyl-2-hydroxy-3-methoxybenzamide	$C_9H_{10}N_2O_4$	*Streptomyces sp.* strain RKND-216	Cytotoxicity and antimicrobial	[27]
3.67	244.1222	Rancinamycin Ib	$C_{11}H_{16}O_6$	*Streptomyces lincolnensis*	Antibacterial	[28]
3.87	212.1490	Bacillusamide B	$C_{10}H_{16}N_2O_3$	*Bacillus sp.*	Antifungal	[29]
4.07	246.1348	Dipyrimicin A	$C_{12}H_{10}N_2O_4$	*Amycolatopsis sp.* strain K16-0194	Antimicrobial and cytotoxicity	[30]
5.03	138.0998	7-Hydroxytropolone	$C_7H_6O_3$	*Pseudomonas donghuensis*	Iron scavenger	[31]
7.53	324.1968	Fluostatin C	$C_{18}H_{12}O_6$	*Streptomyces sp.* strain Acta 1383	Cytotoxicity	[32]
8.29	254.1661	Isoaurostatin	$C_{15}H_{10}O_4$	*Thermomonospora alba*	Topoisomerase inhibitor	[33]
8.47	360.1995	Unknown	-	-	-	-
9.11	402.2320	Unknown	-	-	-	-
9.90	281.2706	Methyl 6-carbamoylphenazine-1-carboxylate	$C_{15}H_{11}N_3O_3$	*Streptomyces diastaticus* strain YIM PH20246	Antimicrobial	[34]

and P1 extracts possessed moderate antibacterial activity against all MDR strains. Crude extracts of M7 isolates exhibited larger inhibition zones in *P. aeruginosa* strain M19, *K. pneumoniae* strain M19, and *B. subtilis* strain M18 than the inhibition zone associated with the P1 extract. The different effects of these soil bacterial extracts on the inhibition of the target bacteria may be influenced by their resistance to antimicrobial agents. MDR bacteria have developed resistance mechanisms against antimicrobial agents, such as through efflux pumps, antibiotic degradation and inactivation, drug target alteration, antibiotic target protection, and reduction in membrane permeability [37]. However, the M7 and P1 crude extracts displayed a broad antibacterial spectrum, as they inhibited the growth of Gram-negative (*K. pneumoniae* strain M19, *P. aeruginosa* strain M19, and *E. coli* strain M4) and Gram-positive (*B. subtilis* strain M18 and MRSA) bacteria.

The investigation of the antibacterial activity of the two selected isolates was then continued using determining the lowest extract concentration required to inhibit (MIC) and kill (MBC) the target bacteria as one of the pharmacological parameters of antibacterial compounds to be used for medicinal purposes. Lower concentrations of this compound are expected to reduce its toxicity in the human body. The MICs of M7 and P1 crude extracts ranged from 312.5 µg/ml to >10,000 µg/ml. The MIC obtained in this study are also higher than those reported in earlier studies. The MICs of extract from *Bacillus safensis* MK-12.1 were 3.12–6.25 mg/ml against antibiotic-resistant strains of *Acinobacter baumanii*, *E. coli*, *P. aeruginosa*, and *S. aureus* [38]. The MICs of the bacterial extracts used in this study are also stronger than that of *Bacillus cereus* extract, which ranges from 8.34 to 33.34 mg/ml against *Shigella dysenteriae*, *Salmonella typhi*, *S. aureus*, *E. coli*, and *Corynebacterium diphtheriae* 8.34–33.34 mg/ml [39]. However, further investigation is necessary to purify the antibacterial compounds present in the M7 and P1 extract. Furthermore, in this study the MBCs of both crude extracts were higher than the MICs, suggesting that higher concentrations were required to completely kill all the target bacteria in the medium. Another study reported that MBC may be equal to or 2–4 fold higher than the MIC [40].

We further investigated the antibiofilm activities of the two potent bacterial extracts. The biofilm structure plays a crucial role in antibiotic resistance mechanisms because it inhibits the penetration of antibiotics and protects microbial cells from the host immunity [41]. In the present study, the M7 and P1 extracts (at a concentration of 2× MIC) showed the best antibiofilm activity against biofilms formed by MRSA and *B. subtilis* strain M18, respectively. Notably, several antibiofilm agents have been previously reported from numerous soil *Bacillus* sp. strains, including *Bacillus* spp. against MRSA and *A. baumanii* biofilms [42], *B. cereus* against MRSA biofilm [43], and *B. cereus* ILBB55 against *S. aureus*, and *P. aeruginosa* biofilms clinical strains with diverse antibiofilm capacities [44]. This is likely because the sources of *Bacillus* spp. isolates were different, thus the active compounds produced by these bacteria exhibit various characteristics and bioactivities.

Isolates M7 and P1 isolates were identified based on the 16S rRNA gene. This gene is used for bacterial identification because it is found in all prokaryotic organisms, contains both variable and conserved regions, and evolves slowly [45]. Sequence alignment of the 16S rRNA gene showed that isolates P1 and M7 were closely related to *B. aerius* strain 24K and *P.* (*Bacillus*) *aryabhattai* strain B8W22, respectively. According to Stackebrandt and Goebel [46], two organisms with a DNA similarity of >97.5% are closely related at the species level. Interestingly, *Bacillus* produces various metabolites that have antibacterial effects, such as bacitracin [47], macrolactin [48], and bacilysin [49]. Thus, the metabolites produced by soil bacteria from Muna Island may be an alternative source of novel antibiotics.

The presence of antibiotic-related genes in both isolates suggests that they may have the potential to produce active molecules. Using a molecular approach, M7 and P1 isolates were shown to contain the *mlnA*, *dfnD*, *baeR*, *srfA*, and *bacD* genes, which are involved in the biosynthesis of macrolactin, difficidin, bacillaene, surfactin, and bacilysin, respectively. These metabolites are primarily produced by *Bacillus* spp., have remarkable biological activity, and act as antibacterial agents [50]. Macrolactin, bacillaene, and difficidin are types of polyketides that inhibit bacterial growth through the inhibition of protein synthesis. These compounds have been reported to show antibacterial activity against pathogens, such as *S. aureus*, *E. coli*, and *P. aeruginosa* [51]. Surfactin is an amphiphatic lipopeptide that exhibits an antibacterial mechanism by destabilizing and interrupting membrane integrity [52]. Surfactin from *B. cerulans* is active against MDR strains, including MRSA, *K. pneumoniae*, and *E. coli* [53]. Bacilysin is a peptide antibiotic that exhibits broad antibacterial properties by interfering with glucosamine synthesis [54]. However, the *NRPS, PKS type 1, dhbE, ituA,* and *fenA* genes were not detected in the M7 and P1 genomes. This suggests that these genes are not involved in the biosynthesis of antibacterial compounds by the two isolates.

To investigate the potency of the most potent extract (M7), the secondary metabolite constituents present in the corresponding extract were identified using LC-MS/MS analysis. The results indicated that the potential extract contained 13 putative compounds as identified in the library database (Fig. 5). Interestingly, three compounds namely cyclo(D-Pro-L-Tyr), marinoquinoline G, and rancinamycin Ib have been reported to exhibit high antibacterial activity against the phytopathogenic bacteria, *Pontibacillus* sp. and *S. aureus*, respectively [24–25,28]. Furthermore, three putative compounds including N-carbamoyl-2-hydroxy-3-methoxybenzamide, dipyrimicin A, and methyl 6-carbamoylphenazine-1-carboxylate have antimicrobial and cytotoxic properties [27,30,34]. Therefore, our results confirm that the antibacterial and antibiofilm properties of the potential M7 extract are likely due to the involvement of these compounds. In addition, the M7 extract contained several compounds that exhibited some potential bioactivities, including 2,6-dimethoxy terephthalic acid (antioxidant), bacillusamide B (antifungal), 7-hydroxytropolone (iron scavenger), fluostatin C (cytotoxicity), and isoaurostatin (topoisomerase inhibitor) [26,29,31–33].

CONCLUSİON

Five of the 15 soil bacterial isolates (33%) exhibited various antagonistic activity against the tested MDR strains.

Among the 15 isolates, M7 and P1 were the most potent isolates, as indicated by their broadest antibacterial spectra against all MDR strains. The crude extracts from these two isolates inhibited all MDR strains. However, the extracts were more active against *E. coli* strain M4, *B. subtilis* strain M18, and MRSA, as indicated by their MIC values. The two selected isolates were identified as *Bacilli*. The presence of metabolite biosynthesis genes, including *mlnA*, *baeR*, *dfnD*, *srfA*, and *bacD*, further suggests the ability of the bacteria to produce antibacterial compounds. These findings demonstrate that naturally occurring *Bacillus* spp. has a significant potential to produce active constituents against bacteria and disrupt their biofilm structure, enabling the discovery of novel antibiotics. Further characterization and structural elucidation of the active compounds are required to expand our knowledge and enable the development of new antibiotic candidates.

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AUTHOR CONTRIBUTIONS

JAP, MEP, and RIA conceived the study. ENWH, JAP, and MEP performed the laboratory experiments. ENWH, JAP, MEP, LOAFH, and JML acquired and analyzed the data. An early version of this manuscript was written by ENWH, and revised by JAP, MEP, RIA, LOAFH, and JML.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

The 16S rRNA sequences of the potential bacteria can be downloaded from the NCBI GenBank database with the accession IDs OR066161.1, and OR066162.1. All other experimental data are included in this article.

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