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Anti-MDR bacterial activity of bacteria associated with sea sponge of *Amphimedon* sp. from Karimunjawa Island, Central Java, Indonesia

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ABSTRACT

Aims: The aim of this study was to evaluate the antibacterial activity of bacteria associated with sea sponge *Amphimedon* sp. against multi-drug resistant (MDR) bacteria that cause wound infections.
Methodology and results: The antibacterial activity was evaluated by the overlay method. Identification of active bacterial symbionts was carried out using the 16S rRNA gene sequence-based method of bacterial identification. The results suggest that one of nine isolates had antibacterial activity against MDR bacteria. Isolate Z9VIII was identified as *Bacillus subtilis* and demonstrated robust antibacterial activity against bacteria: MRSA (11 mm), MDR-AB (17 mm) and CRPA (12 mm).
Conclusion, significance and impact of study: This study concludes that the one of the bacterial species associated with the sea sponge of *Amphimedon* sp. was *B. subtilis*, which has the potential as antibacterial agent against MDR bacteria.

Keywords: Antibacterial activity, *Amphimedon* sp., *Bacillus*, MDR bacteria, sponge, wounds

INTRODUCTION

Wounds are damage to body tissues caused by punctures, cuts and other impacts. Wound infection is one of the health problems caused by the entry of pathogenic microorganisms such as bacteria. Infection can be caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, coagulase-negative *Staphylococcus*, *Klebsiella pneumonia*, *Acinetobacter* sp. and *Proteus mirabilis* (Adhikari *et al.*, 2020). One way to treat wound infections is generally using antibiotics. Antibiotics are drugs used to treat bacterial infections by killing or inhibiting the growth of bacteria (CDC, 2021). The use of antibiotics has marked a turning point in the way that pathogenic bacterial infections are treated in medicine. However, every discovery of antibiotics must be followed by the emergence of resistance. The emergence of bacteria resistant to many antibiotics (multi-drug resistant) is a big challenge in treating infections (Karaman *et al.*, 2020). Over the last few decades, there has been an alarming increase in the prevalence of antibiotic-resistant bacterial strains (Hasan *et al.*, 2016). Multi-drug resistant (MDR) bacteria are defined as bacteria resistant to three or more antibiotics tested *in vitro* (Alemayehu, 2021). WHO has declared dangerous MDR bacterial strains, and a variant of concern includes carbapenem-resistant *Acinetobacter baumanii* (CRAB), carbapenem-resistant *P. aeruginosa* (CRPA), carbapenem-resistant *Enterobacteriaceae* (CRE), extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae*, vancomycin-resistant *Enterococcus faecium* (VRE) and methicillin-resistant *S. aureus* (MRSA)

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(WHO, 2017). These MDR bacterial strains are considered to cause severe infections with varying prevalence globally.

MRSA is a bacterium resistant to β-lactam antibiotics, including the penicillin group (methicillin, oxacillin and nafcillin) (Brooks *et al.*, 2013). MRSA is a bacterium commonly found in infections and is a significant cause of nosocomial infections worldwide. The highest prevalence of MRSA in the world is reported to be in Asia. Most hospitals in Asia are endemic for MRSA, ranging from 28% to >70% (Chen and Huang, 2014). Meanwhile, the resistance of *P. aeruginosa* bacteria to carbapenem antibiotics, such as imipenem and meropenem, is currently a serious problem in treating patients. The emergence of CRPA strains makes antibiotic therapy difficult for patients. CRPA ranks second with high antibiotic resistance, including carbapenems and cephalosporins. Generally, both classes of antibiotics are used to treat MDR bacterial infections (WHO, 2017). The prevalence of CRPA in Taiwan was 15.9%, while other studies report that the prevalence of CRPA in the United States was 60.3% (Cai *et al.*, 2017; Tsao *et al.*, 2018). Meanwhile, the prevalence of CRPA in Asia, which was identified from a total sample of 6349 *P. aeruginosa* isolates from 14 countries in Asia, indicated that 1,198 isolates belong to the CRPA strain (18.9%) and 1,303 MDR-*P. aeruginosa* isolates (20.5%) (Lee *et al.*, 2019). In addition to MRSA and CRPA, infection with MDR-*A. baumanii* (MDR-AB) has become a focus in the world of health. MDRAB has become a health problem in hospitals worldwide and is often associated with nosocomial infections with poorer clinical outcomes in hospitalized patients (Nasr, 2020). Based on the prevalence study of antibiotic-resistant *A. baumanii* with clinical samples obtained from hospital ICU rooms in Southeast Asia, the results were very high, especially for the carbapenem (CRAB; 64.91%) and multi-drug resistant (MDR-AB; 58.51%) groups (Teerawattanapong *et al.*, 2018).

Infections caused by multi-drug resistant (MDR) bacteria have become a health problem worldwide, with a high number of cases, one of which is in Indonesia (Suwantarat and Carroll, 2016). The inappropriate use of antibiotics is one of the causes of bacterial resistance to antibiotics. The emergence of a broad pattern of antibiotic resistance can limit the choice of antibiotics used in the treatment of infections and can have a negative impact on patients (Lai *et al.*, 2014). Cases of MDR bacteria are a major focus of treatment globally, although their distribution varies by region or country. Another study reported that the prevalence of MDR bacterial infection cases in Ethiopia reached 70.5% (Kang and Song, 2013; Alemayehu, 2021).

β-lactam antibiotics are generally used to treat gram-negative bacterial infections, which can cause bacterial resistance to β-lactam. ESBL producing-*Enterobacteriaceae*, especially *E. coli* and *K. pneumoniae*, have increased in recent years (Du *et al*., 2002; Bayraktar *et al.*, 2018). In addition, the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) strains has recently become a serious threat to public health because of the high mortality rate, spread rate and limited drug options in its treatment. Bacterial resistance, especially Gram-negative bacteria, was determined by being included in the list of variants of concern to get the latest antibiotics in their treatment (Nair and Vaz, 2013; WHO, 2017).

To overcome the problem of using antibiotics that can cause antibiotic-resistant bacteria, we need natural ingredients that have the potential and can be developed as antibacterial agents. Natural antibacterial agents can be obtained from seeds (Prastiyanto *et al.*, 2020a; 2021a; 2022a), mushroom (Prastiyanto *et al.*, 2017; 2020b), latex (Prastiyanto *et al.*, 2020c), herbal plants (Prastiyanto *et al.*, 2021b), bacterial isolates from marine organisms (Kusmita *et al.*, 2021; Prastiyanto *et al.*, 2022b) and fruits (Prastiyanto *et al.*, 2020d) even plasma jet (Darmawati *et al.*, 2019). One of the natural ingredients that can be used as an antibacterial is bacteria associated with marine sponges.

In the last few decades, many researchers have been interested in researching bioactive compounds isolated from marine organisms, such as seaweed, algae, cnidarians, sponges, mollusks, marine microorganisms and phytoplankton (Blunt *et al.*, 2018; Carroll *et al.*, 2022). It is known that many marine organisms act as producers of useful bioactive compounds and can be used as alternative antibacterial agents to replace antibiotics. The resulting bioactive compounds have the potential to be effective against pathogens that infect humans (Radjasa *et al.*, 2013; Kusmita *et al.*, 2021; Prastiyanto *et al.*, 2022b). Indonesia is an archipelagic country that is the center of the diversity of coral reefs worldwide, including marine sponges. Marine sponges are one of organisms rich in bioactive compounds with biological activities. The active compounds produced can be used as antioxidants, antibacterial, anti-inflammatory, antifungal, anticancer, antiviral and immunomodulator activities (Abdelmohsen *et al.*, 2017).

Many studies have reported that sponges show potential as antibacterial agents. Compounds derived from marine organisms, including marine sponges, have antibacterial activity in inhibiting the growth of Gram-positive and Gram-negative bacteria and inhibiting the growth of MDR bacteria (Liu *et al.*, 2019). Compounds derived from marine sponges were reported to inhibit the growth of *E. coli* and *S. aureus* bacteria and could accelerate wound healing based on *in vivo* tests of sponge metabolites (Ye *et al.*, 2016). A study reported that three polybrominated diphenyl ether compounds were isolated from the marine sponge *Dysidea granulosa*. These compounds showed strong spectrum antibacterial activity against MRSA, MSSA, *E. coli* and *Salmonella* (Sun *et al.*, 2015).

This study used samples of the marine sponge *Amphimedon* sp. Sponge *Amphimedon* sp. belongs to the Demospongia class, which is reported to contain many bioactive compounds. Alkaloid compounds manzamine and zamamidine D isolated from sponge *Amphimedon* sp., Okinawa, showed activity against bacteria and fungi (Kubota *et al.*, 2017). In addition to containing primary

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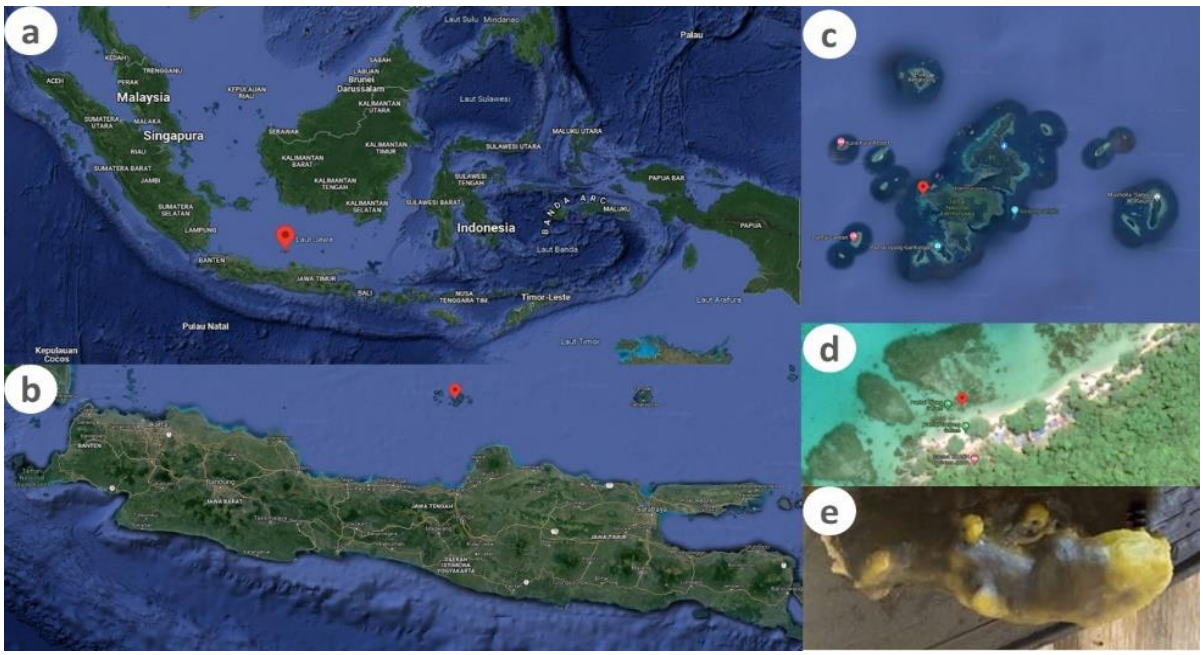

Figure 1: Sampling location on Karimunjawa Island, Tanjung Gelam Beach, Jepara Regency, Central Java, Indonesia. A-D are sampling locations for marine sponges and E is a sample for sponge *Amphimedon* sp. taken from the location point.

and secondary metabolites, there are also bacteria associated with the marine sponge *Amphimedon* sp. Bacteria associated with sponges have been reported to inhibit the growth of pathogenic bacteria in humans (Cita *et al.*, 2017). This study only focused on bacteria associated with marine sponges. This study aimed to isolate bacteria associated with the marine sponge *Amphimedon* sp. and evaluate its antibacterial activity against MDR bacteria that cause wound infections.

MATERIALS AND METHODS

Marine sponge *Amphimedon* sp. sample collection

Sponge samples were collected approximately from a depth of 0.5-1 m from the Karimunjawa Archipelago Sea, Tanjung Gelam Beach, Jepara, Central Java, Indonesia (Figure 1), with coordinates 5°50'22.7"S 110°24'39.8"E. The samples were then stored in sterile bags underwater, in a cooler (4 °C) and transported to the laboratory. Sponges were identified and classified at the Fisheries and Marine Laboratory, Universitas Diponegoro, Semarang, Indonesia.

Isolation of marine sponge *Amphimedon* sp. symbiont bacteria

Sponge samples were processed under aseptic conditions. Samples were cleaned using sterile seawater to remove dirt, plants and epiphytic microorganisms adhering to the surface. Then, the sample was crushed as much as 1 g using a mortar until smooth and put into a 10^{-1} to 10^{-4} differential dilution tube. About 100 µL of the sample was pipetted and spread into a petri dish containing Zobell marine agar (Marine agar 2216E) and then incubated at 37 °C for 2 days. Colonies were selected based on morphological differences. Colonies with different morphologies were transferred to the same medium to obtain pure cultures.

Isolation of MDR bacteria from wound Infection

Multi-drug resistant (MDR) bacteria were isolated from wound infection patients at Tuguredjo General Hospital, Semarang, Central Java, Indonesia. All isolates were identified, and susceptibility patterns were obtained using Vitek®MS (bioM'erieux). MDR bacteria were sub-cultured on BAP media containing 5% sheep's blood for Gram-positive bacteria and Mac Conkey's medium for Gram-negative incubated at 35 ± 2 °C for 24 h. MDR bacterial colonies were suspended and adjusted to the standard McFarland 0.5 (5 × 10^8 CFU/mL) using a McFarland Densitometer.

Antibacterial activity test

Screening to determine antibacterial activity against MDR bacteria was carried out using the overlay method (Radjasa *et al.*, 2013). Pure cultures were inoculated ± 1 cm^2 on Zobell marine agar medium in triplicate. The surface of the medium was covered with Muller Hilton soft agar (0.3% (w/v) containing 1% (w/v) NaCl and 1% (v/v) of MDR *E. coli*, MDR *K. pneumoniae*, CRPA, MRSA and MDR *A. baumanii* after the bacteria had grown, which

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typically takes 1-7 days depending on the bacterial growth rate. After that, all plates underwent a 24 h aerobic incubation at 35 ± 2 °C. The diameter of the inhibition zone around the bacterial isolates, expressed in millimeters, was used to calculate the antibacterial activity of the isolates. The following equation was used to determine the inhibition index (II) (Apsari *et al.*, 2019).

Inhibition index (II) = [Inhibition zone diameter (mm) - Colony diameter (mm)]/Colony diameter (mm)

Molecular identification of marine sponge symbiont bacteria

DNA was extracted from bacterial cells using Kit Presto™ Mini gDNA Bacteria Kit according to the appropriate protocol in the manufacturer's instructions, with a final elution volume of 50 µL. The extracted DNA was stored at 4 °C until required for PCR. The concentration of bacterial DNA used was 584 ng/µL. This step used 2 µL 16S primer gen rRNA 27F '5-AGAGTTTCMTGGCTCAG-3' and 2 µL 16S rRNA primer gen 1492R '5-GGTTACCTTGTTACGACTT-3'. The final concentration of 10 µM primer was 10 µM. Mixing reagents were GoTaq(R), Green Master Mix, 100 Reaction® as much as 12.5 µL, DNA template 1 µL, forward primer 2 µL, reverse primer 2 µL, Nucklease Free Water 7.5 µL. The volume of each tube was 25 µL. PCR was conducted using Biometra T Personal Thermocycler. The amplification conditions for the second PCR were denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 2 min. The PCR products were separated on 2% agarose gel and the DNA bands were visualized with Fluoro Viu. Genetika Science Jakarta performed PCR product sequencing to analyze the 16S rRNA gene sequence. The tracking results were obtained through the GenBank Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI), National Institute for Health, the USA, on the website www.ncbi.nlm.nih.gov.

Phylogenetic analysis

MEGA X software was used for phylogenetic analysis. The results of 16S rRNA gene sequencing were aligned using ClusterW. The phylogenetic tree was determined by the neighboring pooling method with the Tamura-Nei model and supplemented by non-parametric bootstrap analysis (1000 datasets) of the 16S rRNA gene sequence. The results showed the phylogenetic relationships of the closely related strains database available on the NCBI GenBank.

RESULTS AND DISCUSSION

MDR bacteria from wound

The results of identification and bacterial susceptibility tests from wounds showed that all strains were MDR (ESBL-producing *E. coli*, ESBL-producing *K. pneumonia*, MRSA, MDRAN and CRPA) (Table 1). Genus *Staphylococcus* and *Pseudomonas* (Rahim *et al.*, 2016), *S. aureus*, *P. aeruginosa*, *E. coli*, *P. mirabilis* and *A. baumanii/haemolyticus* bacteria are often found in wounds (Puca *et al.*, 2021). The results of another study from wound samples at the University Medical Center (Lubbock, TX), Texas, United States, showed that the 4 multi-drug resistant (MDR) bacteria commonly found in wound samples were *S. aureus*, *Enterococcus*, *P. aeruginosa* and *E. coli* (Trivedi *et al.*, 2014).

***Amphimedon* sp. symbiont bacteria**

Nine bacterial isolates were obtained from the isolation of the marine sponge symbiont *Amphimedon* sp. (Figure 2). The results showed morphological differences in each bacterial isolate. The 9 isolates included two Gram-positive *cocci*, one Gram-negative *coccus*, three Gram-positive *bacilli*, two Gram-negative *bacilli* and one Gram-positive endospores-forming *Bacillus* isolates (Table 2).

Table 1: Bacteria from wound samples.

Species	Antibiotic resistance pattern	MDR name
Escherichia coli	ampicillin, cefazolin, ceftazidime, ceftriaxone, cefepime, aztreonam, ciprofloxacin, nitrofurantoin sulfamethoxazole	ESBL-producing *E. coli*
Klebsiella pneumoniae	ampicillin, sulbactam, tazobactam, cefazolin, ceftazidime, ceftriaxone, cefepime, aztreonam, ertapenem, meropenem, ciprofloxacin, sulfamethoxazole	ESBL-producing *K. pneumoniae*
Staphylococcus aureus	benzylpenicillin, oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, nitrofurantoin, sulfamethoxazole	MRSA
Acinetobacter baumannii	ampicillin, sulbactam, tazobactam, cefazolin, ceftazidime, ceftriaxone, cefepime, aztreonam, meropenem, amikacin, gentamicin, ciprofloxacin, sulfamethoxazole	MDRAB
Pseudomonas aeruginosa	ampicillin, sulbactam, tazobactam, cefazolin, ceftazidime, ceftriaxone, cefepime, aztreonam, meropenem, amikacin gentamicin, ciprofloxacin, tigecycline, nitrofurantoin, sulfamethoxazole	CRPA

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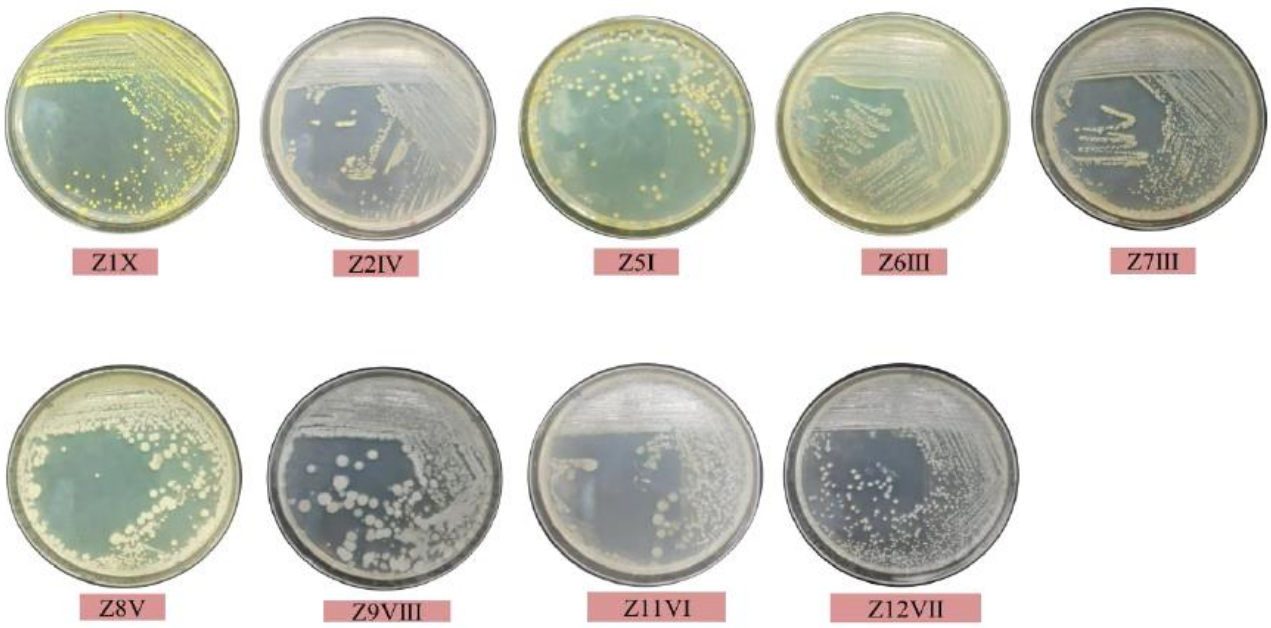

Figure 2: Macroscopic colony morphology of symbiont bacteria isolates *Amphimedon* sp. on Zobell marine agar.

Table 2: Colony morphology and Gram staining of bacterial isolates from sponge *Amphimedon* sp.

Isolate code	Colony morphology					Gram staining
	Shape	Margin	Elevation	Color	Size (mm)	
Z1X	Circular	Entire	Convex	Yellow	1	*Coccus*, Gram-positive
Z2IV	Filamentous	Undulate	Umbonate	Yellow	1	*Bacillus*, Gram-negative
Z5I	Circular	Entire	Convex	Yellow	1.5	*Bacillus*, Gram-negative
Z6III	Circular	Entire	Convex	Yellow	0.5	*Coccus*, Gram-negative
Z7III	Circular	Entire	Convex	Brown	0.5	*Coccus*, Gram-positive
Z8V	Irregular	Undulate	Flat	Cream	2	*Bacillus*, Gram-positive
Z9VIII	Irregular	Undulate	Flat	White	3	*Bacillus*, Gram-positive
Z11VI	Irregular	Entire	Flat	White	3	*Bacillus*, Gram-positive, Endospore-forming
Z12VII	Circular	Entire	Convex	White	1	*Bacillus*, Gram-positive

Table 3: Antibacterial activity of isolates of symbiont *Amphimedon* sp. against MDR bacteria.

Isolate	MDR				
	ESBL-producing *E. coli*	ESBL-producing *K. pneumoniae*	MRSA	MDRAB	CRPA
	II (mm)	II (mm)	II (mm)	II (mm)	II (mm)
Z1X	-	-	-	-	-
Z2IV	-	-	-	-	-
Z5I	-	-	-	-	-
Z6III	-	-	-	-	-
Z7III	-	-	-	-	-
Z8V	-	-	-	-	-
Z9VIII	-	-	11	17	12
Z11VI	-	-	-	-	-
Z12VII	-	-	-	-	-

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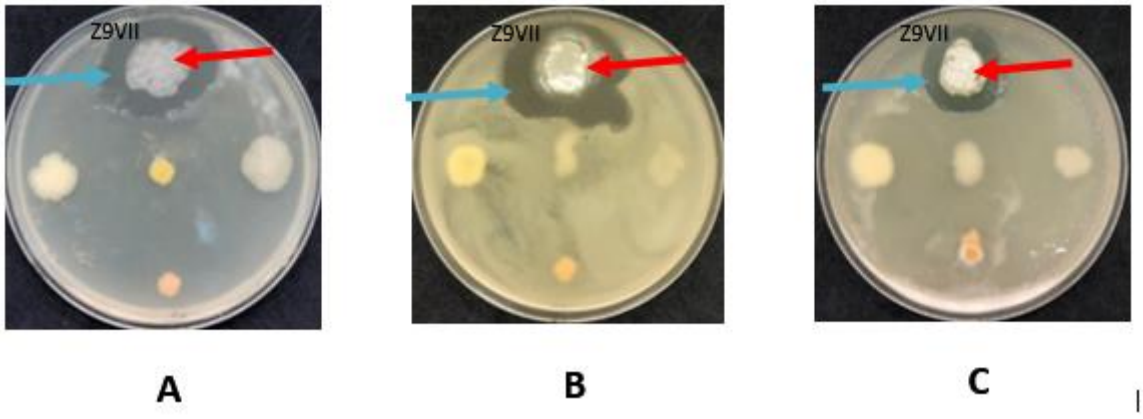

Figure 3: Inhibition zone of *Amphimedon* sp. against MDR bacteria. (A) MRSA, (B) MDR-AB and (C) CRPA. Blue arrow: Inhibition zone; red arrow: Bacterial isolate colonies.

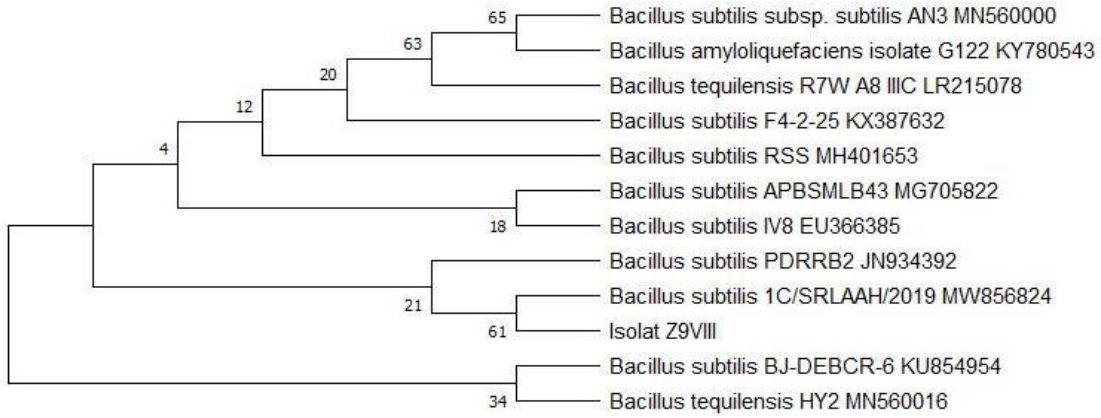

Figure 4: The bacterial strain phylogenetic tree associated with this study is the Tamura-Nei model of the 16S rRNA gene sequence.

Antibacterial activity against MDR bacteria

The antibacterial activity of the symbiont *Amphimedon* sp. against MDR bacteria isolated from the wound indicated the presence of inhibition. The inhibition zone is a qualitative test to determine the ability of antibacterial agents to inhibit the growth of microorganisms, especially pathogenic bacteria. The results showed that one isolate from 9 isolates of the symbiont bacteria *Amphimedon* sp. showed antibacterial activity against MDR bacteria. Z9VIII isolate showed powerful antibacterial activity with respective inhibition zone indices against MRSA (11 mm), MDR-AB (17 mm) and CRPA (12 mm) (Table 3 and Figure 3).

Molecular identification of the symbiont bacteria *Amphimedon* sp.

Isolate Z9VIII was selected for molecular identification based on the antibacterial activity against MDR shown by this isolate. The phylogenetic tree reveals that the symbiont *Amphimedon* sp. (Z9VIII) was found to be closely related to *B. subtilis* Accession number MW856824 (Figure 4).

In research conducted by Matobole *et al.* (2017), thirty-five isolates showed antibacterial activity and twelve showed antibacterial activity against MDR bacteria. The diversity of bacteria associated with marine sponges varies greatly between sponge species (Webster and Taylor, 2012; Haber and Ilan, 2014). Bacterial isolates demonstrated very strong antibacterial activity against MDR bacteria isolated from wound patients with inhibition zone index against MRSA (11 mm), MDR-AB (17 mm) and CRPA (12 mm), respectively (Table 3 and Figure 3). The results of this study follow previous studies on the biological activity of *Amphimedon* sp. extract. Sponge *Amphimedon* sp. extracted with ethanol solvent could inhibit the growth of *E. coli, P. aeruginosa* and *Enterococcus faecalis* bacteria with inhibition zone sizes ranging from 7.0 mm to 8.5 mm (Lhullier *et al*., 2020). In addition, secondary metabolites produced by *Amphimedon* sp. in the form of halitoxins/amphitoxins compounds play an active role as antifungals and can inhibit the growth of *Aspergillus flavus* (Arevabini *et al.*,

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2014). Many studies have reported that marine natural ingredients (sponges, algae, seaweed, cnidarians, mollusks and phytoplankton) are rich in bioactive compounds that can act as antibacterial, antifungal, antiparasitic, antiviral and anti-inflammatory agents. These bioactive compounds are in the form of primary metabolites, secondary metabolites and microorganisms associated with these marine organisms. This research is in line with previous studies which reported that microorganisms associated with the marine sponge *Amphimedon* sp. showed antibacterial activity against *S. aureus* NCTC 8325, antifungal against *Candida albicans* 5314 and anti-*Trypanosoma* (Alkhalifah, 2021). Another related study reported that most microorganisms isolated from the sponge *Amphimedon* sp. belong to the genus *Bacillus*. *Bacillus* sp. and *Bacillus pumilus* showed high antibacterial activity against MDR-*Pseudomonas* sp. and weak against ESBL-*K. pneumoniae* and moderate to MRSA. Meanwhile, *Bacillus safensis* could inhibit the growth of MDR-*Pseudomonas* sp. and ESBL-*K. pneumoniae* with moderate intensity and inhibit MRSA with weak intensity. *Bacillus* isolated from the sea has the potential to produce bioactive compounds as antibacterial agents (Aboul-Ela *et al.*, 2019).

Several studies reported the antibacterial potential of the genus *Bacillus*. Based on genomic analysis, *Bacillus* sp. produces metabolites that have the potential as antibacterials, such as difficidin, macrocyclic, macrolactin, bacillaene and other bioactive compounds. Pulcherriminic acid is a cyclic dipeptide synthesized by the genus *Bacillus*. Pulcherriminic acid is bacteriostatic by scavenging Fe^{3+} ions to form reddish-brown *Pulcherrimin*. In a low iron environment, microbes that produce pulcherriminic acid can compete with other microbes for iron and therefore, in low iron environmental conditions, other microbes do not obtain inorganic salts, especially iron (Kántor *et al.*, 2015). Difficidin compounds are products identified from *Bacillus*, which are associated with marine organisms. *Bacillus amyloliquefaciens* MTCC12713 is one of the difficidin producers. Difficidin has been reported to exhibit strong antibacterial activity against MRSA, VRE, *K. pneumonia, E. coli* and *S. pyogenes* (Chakraborty *et al.*, 2021). Macrolactin A, macrolactin Q and macrolactin W chemicals were produced during fermentation by *Bacillus* sp. 09ID194 that was found in the sea. These three substances effectively combat both Gram-positive and Gram-negative harmful microorganisms (Mondol *et al.*, 2011). Volatile organic compounds (VOC) produced by *Bacillus* sp. BO53 is associated with marine organisms. Volatile bioactivity was tested *in vitro* against antibiotic-resistant human pathogenic bacteria, MRSA, *A. baumanii* and *P. aeruginosa* (Garrido *et al.*, 2020). Surfactin, produced by *Bacillus* sp. CS30, isolated from the sea, was reported to act as an antifungal and antibacterial compound. The mechanism of surfactin action is related to an increase in reactive oxygen species (ROS) and causes damage to the cell wall and cytoplasm (Wu *et al.*, 2019). In addition, *Bacillus subtilis* MSH1 was reported to produce antibacterial surfactin (Isa *et al.*, 2017).

CONCLUSION

This study concludes that the bacteria associated with the sponge of *Amphimedon* sp. that have been identified are related to *Bacillus subtilis* and have the potential as antibacterial agents against MDR bacteria.

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