Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(4): 2775 – 2787 (2025)
www.ejabf.journals.ekb.eg

Antibacterial Activity of Tunicate-Associated Bacteria from Karimunjawa Sea Against Aquaculture Pathogens

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ARTICLE INFO

Article History:
Received: May 15, 2025
Accepted: July 28, 2025
Online: Aug. 20, 2025

Keywords:
Antibacterial,
Associated bacteria,
Bacillus haynesii,
Tunicate

ABSTRACT

The primary challenge in shrimp aquaculture lies in pathogenic bacterial infections, which frequently result in mass mortality and substantial economic losses. Environmentally sustainable antibacterial agents are urgently needed to mitigate infections caused by *Pseudomonas aeruginosa* and *Escherichia coli*, both of which are known to induce hepatopancreatic and muscle tissue infections in shrimp. This study aimed to assess the antibacterial potential of tunicate-associated bacteria and to identify candidate antibacterial agents effective against *P. aeruginosa* and *E. coli*. A descriptive–exploratory approach was employed, including bacterial isolation using the spread plate method, purification via the streak plate method, antibacterial activity screening through agar plug diffusion, and molecular identification based on 16S rRNA gene sequencing. A total of 51 bacterial isolates were obtained from six tunicate samples, with 9.8% exhibiting antibacterial activity against at least one test pathogen. The most significant activity was observed in bacteria associated with *Didemnum* sp., which demonstrated an inhibition zone diameter of 11.2mm against *P. aeruginosa*. These findings suggest that bacterial diversity does not directly correlate with bioactive compound diversity, as the production of secondary metabolites appears to be driven by specific microbial interactions. Among the isolates, *Bacillus haynesii* exhibited the highest antibacterial activity, highlighting its potential as a promising candidate antibacterial agent for application in shrimp aquaculture.

INTRODUCTION

Karimunjawa National Park is part of the Coral Triangle, which hosts the highest level of marine biodiversity in the world and serves as a key habitat for various marine organisms **(Campbell *et al.*, 2013)**. The Karimunjawa Sea is a significant center for ecological studies and the exploration of bioactive compounds from marine invertebrates **(Kristiana *et al.*, 2017)** including tunicates **(Hendrawati *et al.*, 2024)**. Ascidians,

belonging to the phylum *Tunicata*, are sessile and soft-bodied organisms, making them vulnerable to predation and spatial competition. As filter feeders, ascidians are constantly exposed to diverse microbial communities, increasing their susceptibility to infections and pathogens. These organisms rely on the production of secondary metabolites as a primary defense mechanism **(Tianero *et al.*, 2015)**. These metabolites not only protect ascidians in their natural habitat but also hold considerable potential in pharmaceutical and biotechnological applications.

Ascidians are recognized as a promising source of bioactive compounds with antibacterial properties, capable of producing cytotoxic compounds effective against pathogenic bacteria affecting both humans and aquatic species **(Chen *et al.*, 2019)**. However, the concentration of these compounds in macroorganisms is often extremely low **(Qian *et al.*, 2007)**. Harvesting and culturing ascidians from the wild is challenging, and large-scale biomass extraction conflicts with marine conservation principles **(Bara *et al.*, 2015)**. A more sustainable alternative is the exploration of symbiotic bacteria associated with ascidians, which have been shown to produce secondary metabolites with antibacterial activity similar to those of their hosts. Metabolites such as terpenoids, meroterpenoids, and alkaloids from ascidian-associated bacteria have been reported to exhibit natural antibacterial activity **(Liu *et al.*, 2021)**.

The use of natural antibacterial agents is increasingly essential in the aquaculture industry, particularly in shrimp farming, which represents a high-value economic asset, especially in developing countries such as Indonesia **(Wati, 2018)**. Shrimp production is often constrained by pathogenic infections, notably from *Pseudomonas aeruginosa* and *Escherichia coli*. Commonly, these bacteria are part of the normal shrimp microflora **(Ismail *et al.*, 2024)**. However, under certain stress conditions, they can become opportunistic pathogens. *P. aeruginosa* is capable of invading shrimp tissues and proliferating extensively, leading to infections in muscle and hepatopancreatic tissues **(Ramalingam & Ramarani, 2007)**. Both bacteria also contribute to decomposition processes that accelerate spoilage and reduce product quality **(Cholewińska *et al.*, 2022)**. Therefore, exploration of new antibacterial sources in aquaculture is needed to address this issue. One underexplored source is ascidians. This study investigated the antibacterial potential of tunicate-associated bacteria to address pathogenic infections in shrimp aquaculture.

MATERIALS AND METHODS

Sampling procedure

The materials used in this study included tunicate samples collected from Menjangan Kecil, Karimunjawa Sea, at coordinates 5°49′09″S and 110°24′32″E, using SCUBA diving and snorkeling equipment (Fig. 1). Whole-body tunicates were collected to ensure the inclusion of both internal and external structures. Samples were detached

from the substrate using sterile knives or scissors and rinsed with seawater to remove sediment and epibionts. Each sample was placed in a sterile zip-lock plastic bag pre-filled with seawater to maintain aerobic conditions and to prevent degradation of host tissue and associated microbiota. Samples were labeled with unique codes for identification and were stored in a cool box containing ice, ensuring the viability of bacterial communities until further processing in the laboratory. All procedures were carried out under aseptic conditions to minimize the risk of contamination **(Ayuningrum *et al.*, 2019)**.

Sampling site

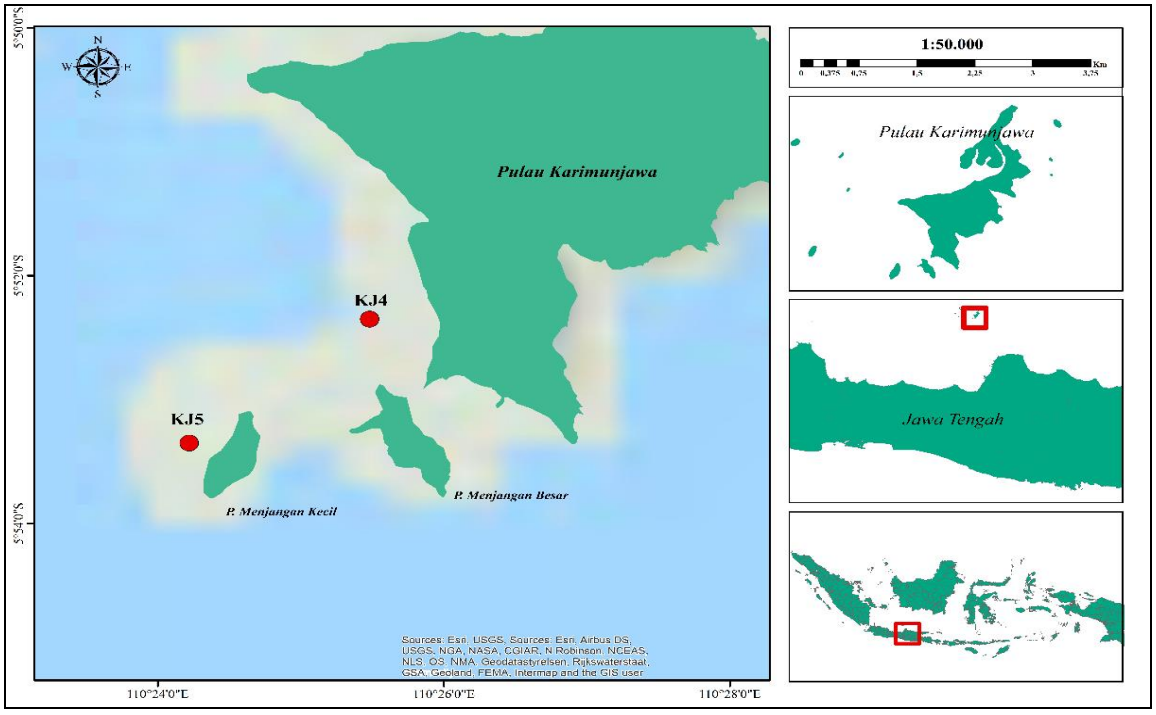

Fig. 1. Map of sampling locations

KJ4 and KJ5: Tunicate sampling sites. KJ4 is in Menjangan Besar island, meanwhile KJ5 is in Menjangan Kecil island.

Isolation and purification of tunicate-associated bacteria

Tunicate samples were rinsed with sterile seawater to remove epibionts, cut into small pieces, and weighed to 1g. The samples were crushed using a sterile mortar and pestle for 2–3 minutes, then homogenized in 10mL of sterile seawater. The homogenate was serially diluted to 10^{-3} by transferring 1mL of the suspension into 9mL of sterile seawater at each dilution step. Subsequently, 100µL from the 10^{-2} and 10^{-3} dilutions were inoculated onto agar plates using the spread plate method. Fast-growing tunicate-associated bacteria were incubated for 2 days, whereas slow-growing bacteria were incubated for 4 days at 24 ± 2°C **(Leal *et al.*, 2014)**.

Bacterial isolation and purification were carried out on Zobell Marine Agar 2216 (HiMedia), a medium optimized for the cultivation and characterization of marine

bacteria **(Merlin *et al.*, 2022)**. Colonies were selected based on morphological characteristics—size, color, shape, and elevation—to maximize diversity. Each distinct colony was purified twice using the streak plate method. Subculturing was repeated until pure colonies with uniform morphology were obtained, ensuring single-strain isolates free from contamination.

Preparation of pathogenic test organisms

Pathogenic test organisms included *Pseudomonas aeruginosa* and *Escherichia coli*, both Gram-negative bacteria sourced from the pathogen culture collection at BBPBAP, Jepara. Strains were revived on Nutrient Agar (NA) plates and incubated for 24 hours at room temperature. A single colony of each strain was inoculated into 10 mL of Nutrient Broth (NB) medium and incubated on a shaker for 24 hours. The bacterial suspensions were then diluted in sterile NB medium and adjusted to a turbidity equivalent to the 0.5 McFarland standard (≈1 × 10^8 CFU/mL) **(Ayuningrum *et al.*, 2019; Hendrawati *et al.*, 2024)**.

A total of 0.1 mL of each standardized suspension was spread evenly over the surfaces of sterile Zobell agar plates to ensure uniform lawn formation, which served as the test medium for antibacterial activity assays.

Antibacterial activity assay

Antibacterial activity was evaluated using the agar plug diffusion method. Pure isolates of tunicate-associated bacteria were incubated on sterile agar plates for 6 days at room temperature. Agar plugs (±6 mm diameter) were aseptically cut using sterile pipette tips and transferred onto plates previously inoculated with the test pathogens.

Each bacterial isolate was tested in triplicate to ensure reproducibility **(Ramesha *et al.*, 2020)**. The plates were incubated at 37°C for 24 hours. Antibacterial activity was indicated by the presence of clear inhibition zones around the agar plugs, and the diameters of these zones were measured using a digital caliper.

The inhibition zone was calculated using this formula **(Bnfaga *et al.*, 2023**):

ZOI = Φ Clear Zone – Φ Agar Plug

Note:

ZOI = Zone of Inhibition (mm)

Φ = Diameter (mm)

The resulting zone of inhibition (ZOI) reflects the antibacterial activity of the bacterial isolate based on the extent of growth inhibition of the pathogenic test bacteria. According to **Ouchari *et al.* (2019)**, the inhibition zones were categorized into four intensity levels: very strong (ZOI > 20 mm), strong (ZOI between 10–20 mm), moderate (ZOI between 5–10 mm), and no response (ZOI < 5 mm).

RESULTS

1. Isolation and purification of tunicate-associated bacteria

Based on the results of this study, a total of 51 bacterial isolates were obtained from six tunicate samples, including *Clavelina robusta*, *Didemnum* sp., *Aplidium* sp. (1), *Aplidium* sp. (2), *Aplidium breviventer*, and one unidentified species (Fig. 2). The sample of *Clavelina robusta* was collected from Menjangan Besar Sea, while the remaining five samples were obtained from different sites around Menjangan Kecil Sea.

Fig. 2. Morphological identification of tunicate samples (*Clavelina robusta*, *Didemnum* sp., *Aplidium* sp. (1), *Aplidium* sp. (2), *Aplidium breviventer*, and unidentified species respectively)

As shown in Fig. (3), the diversity of bacterial isolates varied among the tunicate samples. The highest number of isolates was obtained from *Clavelina robusta* collected from Menjangan Besar, with a total of 13 isolates. In contrast, the lowest number of isolates was derived from *Didemnum* sp. (1) from Menjangan Kecil, yielding only 3 isolates. Other samples exhibited a variable range of isolate numbers, between 6 and 11.

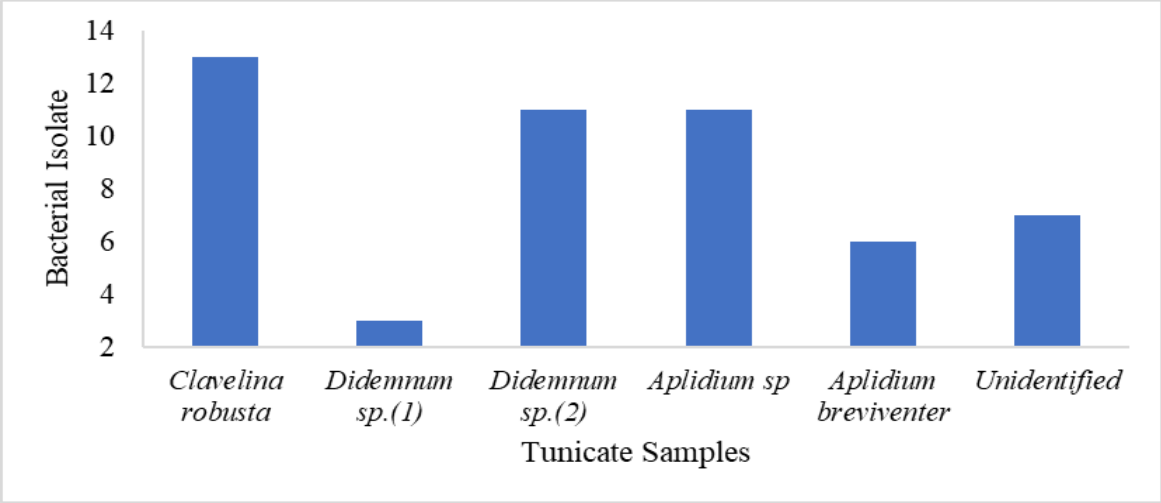

Fig. 3. Number of bacterial isolates per tunicate samples

2. Antibacterial activity of tunicate-associated bacteria

The antibacterial activity assay of the bacterial isolates associated with tunicates against *Pseudomonas aeruginosa* and *Escherichia coli* revealed that most isolates did not exhibit significant antibacterial effects. The isolate coded KJ5-02$^{(-2)}$/3, derived from *Didemnum* sp., demonstrated the highest antibacterial activity and was the only isolate categorized as having "strong" activity against *P. aeruginosa*. The isolate with the highest antibacterial activity, coded KJ5-02$^{(-2)}$/3, was obtained from *Didemnum* sp. (2).

Table 1. Results of antibacterial activity assay against *P. aeruginosa*

No	Isolate Code	Diameter (mm)				Species
		1st Tested	2nd Tested	3rd Tested	Average±SD	
1.	KJ5-08 $^{(-3)}$/4	4,3	3,6	2,2	3,3±1.1	*Aplidium breviventer*
2.	KJ5-01 $^{(-1)}$/2	5,1	2,8	-	3,9±1.6	*Didemnum* sp. (1)
3.	KJ5-02 $^{(-2)}$/3	10,7	11,2	11,6	11,2±0.4	*Didemnum* sp. (2)

Table 2. Results of antibacterial activity assay against *E. coli*

No	Isolate Code	Diameter (mm)				Species
		1st Tested	2nd Tested	3rd Tested	Average±SD	
1.	KJ5-07 $^{(-1)}$/2	4,3	2,1	-	3,2±1.5	*Aplidium* sp.
2.	KJ5-02 $^{(-}$	4,1	1,9	-	3,0±1.5	*Didemnum* sp. (2)

	[3)]/4					
3.	KJ5-02 [(-2)]/3	2,8	5,6	-	4,2±1.9	*Didemnum* sp. (2)

The antibacterial activity was observed exclusively in isolates derived from tunicates of the genera *Didemnum* and *Aplidium*. Isolates from other tunicate species did not exhibit any detectable antibacterial activity.

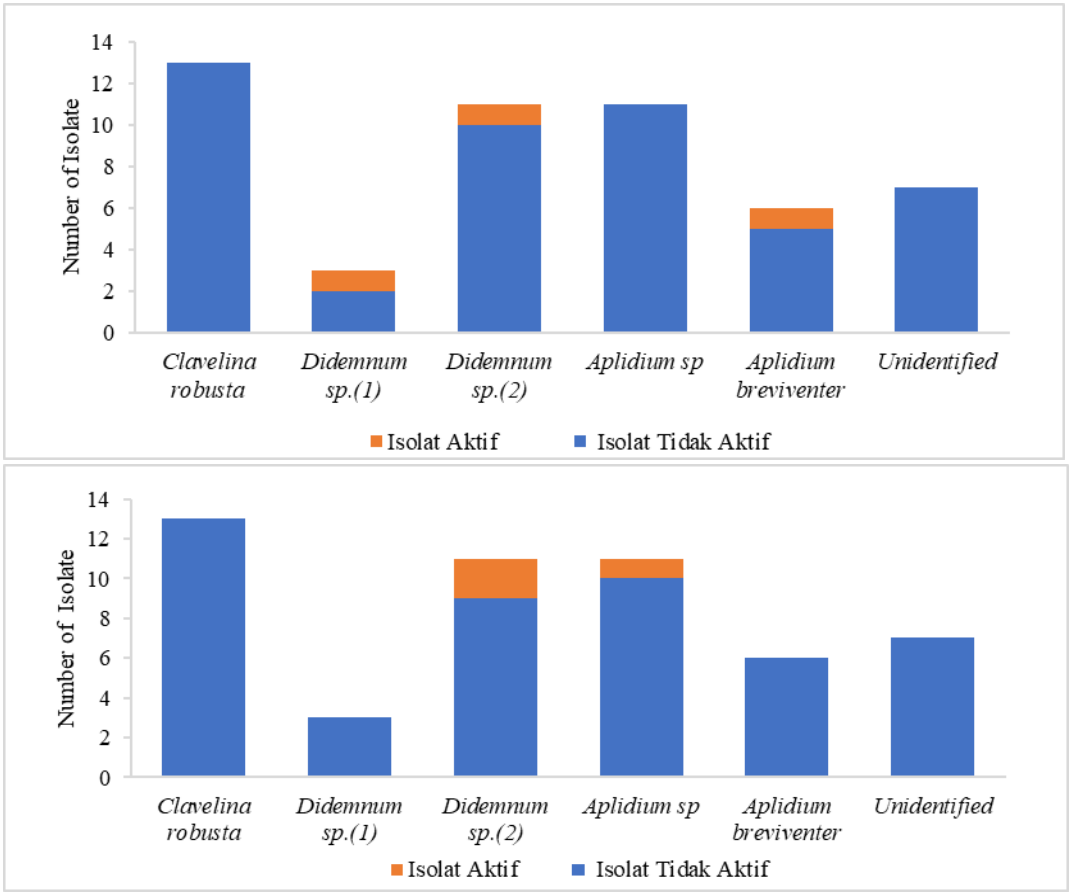

Fig. 4. Comparative antibacterial activity of bacterial isolates against *P. aeruginosa* and *E. coli* per tunicate species

Based on the antibacterial assay, 90.2% of the 51 bacterial isolates associated with tunicates exhibited no antibacterial activity against either *P. aeruginosa* or *E. coli*. In total, only 9.8% of isolates displayed inhibitory effects against at least one of pathogens.

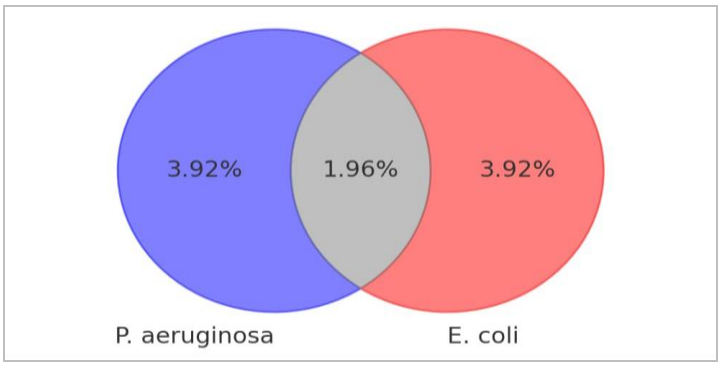

Fig. 5. Percentage of antibacterial activity against both test bacteria

Identification of potential isolate

Morphological identification was performed on the bacterial isolate associated with tunicates that exhibited the highest antibacterial activity. Based on macroscopic observations, the KJ5-02$^{(-2)}$/3 isolate displayed cream pigmentation. The colony was circular in shape, with irregular margins and flat elevation. Microscopically, the isolate appeared as a single rod-shaped (monobacillus) bacterium and was classified as Gram-positive. Molecular analysis revealed that isolate KJ5-02(-2)/3 shared 99% sequence similarity with *Bacillus haynesii*.

Table 3. Results of homology search using the BLAST system

No	Isolate Code	Result	Query Cover	Percent Identify	E Value	Length (bp)
1	KJ5-02$^{(-2)}$/3	*Bacillus haynesii*	99%	99,59%	0.0	1.500

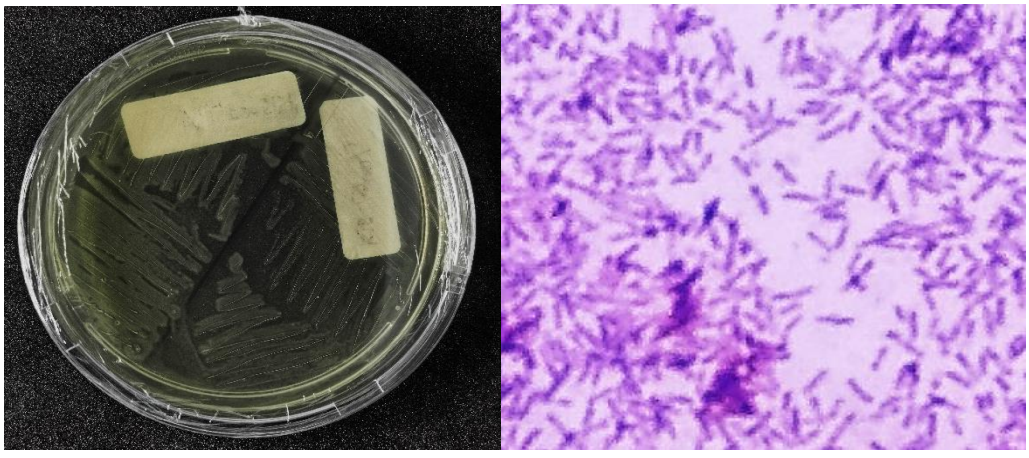

Fig. 6. Morphological characteristics of the potential isolate KJ5-02$^{(-2)}$/3

DISCUSSION

The diversity of bacterial associations found in tunicates in this study was relatively high, indicating that these invertebrates serve as a rich reservoir for microbial communities. A total of 51 bacterial isolates obtained from six tunicate species suggests that each species harbors bacteria with distinct characteristics. This phenomenon can be explained by the complex interactions between tunicates as hosts and their associated bacteria. As filter feeders, tunicates possess a branchial basket—a specialized anatomical structure that allows the filtration of water and the capture of organic materials and microorganisms, which serve as a nutritional source **(Gordon *et al.,* 2020)**. Bacteria trapped during this filtration process create a microhabitat conducive to the growth of bacterial communities.

Although the bacterial communities associated with tunicates are highly diverse, studies have shown that this diversity is not correlated with geographic location or latitudinal gradients of their habitats. It has been demonstrated that the diversity of tunicate-associated bacteria remains relatively consistent regardless of sampling location or habitat type **(Tianero *et al.,* 2015)**. For instance, bacteria associated with *Styela plicata* in California were found to share a high degree of similarity with samples collected from the same species in the Mediterranean Sea **(Erwin *et al.,* 2013)**. Similarly, bacteria associated with *Lissoclinum badium* from Papua New Guinea exhibited high similarity with samples from the Great Barrier Reef.

Each tunicate species tends to form specific symbiotic relationships with various bacteria. However, comparing the bacterial diversity of each species when cultured in the laboratory is particularly challenging **(Riyanti *et al.,* 2020)**. This difficulty arises because culture media and laboratory conditions significantly influence bacterial growth. Some bacteria may require specific bioactive compounds or enzymes that are only available in their natural habitat, while others depend on symbiotic interactions with tunicates or other bacteria that cannot be replicated *in vitro*. For example, the Zobell medium and laboratory conditions used in this study may have supported the growth of bacteria associated with *Clavelina robusta* but were likely suboptimal for the cultivation of bacteria associated with *Didemnum* sp. (1).

Antibacterial capability appears to be more strongly influenced by specific bacterial interactions that drive secondary metabolite production rather than overall bacterial diversity. In this study, 9.8% of the 51 tunicate-associated bacterial isolates exhibited activity against at least one test pathogen. This proportion is lower than that reported by Meena *et al.*, who found that 12.96% of 298 tunicate-associated isolates were active against at least one pathogen **(Meena *et al.,* 2021)**. In general, tunicates harbor highly diverse microbiomes, but only one or two specific bacterial species typically contribute to pathogen defense via metabolite production **(Tianero *et al.,* 2015)**. A similar pattern was observed by Evans *et al.*, who explained that although tunicates host numerous bacterial

taxa, only a few dominant ones make significant functional contributions **(Evans *et al.*, 2017)**. This observation is further supported demonstrating that specific tunicate-associated bacteria are involved in nitrogen recycling, heavy metal sequestration, pathogen protection, or antifouling activity **(Liu *et al.*, 2021)**. Thus, although only a small fraction of bacteria demonstrate significant bioactivity, tunicate-associated microbes remain a vital source for the discovery of antibacterial compounds.

The antibacterial activity of tunicate-associated bacteria varied across species. *Clavelina robusta* yielded the highest bacterial isolate diversity, yet none of its associated bacteria exhibited antibacterial activity against the test pathogens. In contrast, *Didemnum* sp. (1), which had the lowest bacterial isolate diversity, produced one isolate with antibacterial activity. These findings suggest that bacterial diversity does not directly reflect the diversity of bioactive compounds produced. This observation aligns with the findings of **Tianero *et al.* (2015)**, who reported that despite high tunicate-associated bacterial diversity, there is no direct correlation between microbial diversity and secondary metabolite diversity. Similarly, it was noted that *S. rubra*, which had the highest microbial diversity, exhibited lower metabolite diversity, whereas *A. solidum* produced the highest metabolite diversity but yielded relatively few culturable microbes **(Buedenbender *et al.*, 2017)**.

Phylogenetic analysis based on 16S rRNA sequencing revealed that the most active isolate in this study shared 99% similarity with *Bacillus haynesii*. This bacterium belongs to the phylum Firmicutes, a group characterized by thick peptidoglycan-rich cell walls and predominantly Gram-positive properties. This result is consistent with that of **Meena *et al.* (2021)**, who reported that Firmicutes, particularly the genus *Bacillus*, is the most dominant bacterial group associated with tunicates. *Bacillus* spp. derived from tunicates are well known for their high metabolic activity and involvement in degradation processes and biogeochemical cycles. **Riyanti *et al*. (2020)** further demonstrated that *Bacillus* spp. possess diverse biosynthetic gene clusters (BGCs) responsible for the synthesis of secondary metabolites. The presence of BGCs reflects the potential of *Bacillus* to produce metabolites with antimicrobial, antioxidant, and anti-inflammatory activities **(Lata & Gond, 2025)**.

CONCLUSION

Based on this experimental result, a total of 51 bacterial isolates were obtained from six samples of tunicates, including *Clavelina robusta*, *Didemnum* sp., *Aplidium* sp., and *Aplidium breviventer*. The bacterial isolate coded KJ5-02$^{(-2)}$/3 from the species *Didemnum* sp. exhibited the highest antibacterial activity, as indicated by an inhibition zone diameter of 11.2mm. Molecular identification using BLAST revealed that the isolate KJ5-02$^{(-2)/}$3 was identified as *Bacillus haynesii* with 99.59% percent identity.

ACKNOWLEDGEMENT

This research was fully funded by Faculty of Fisheries and Marine Science, Universitas Diponegoro under the contract number 34/UN7.F10/PM/IX/2023.

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