

Potential of antagonistic activity from associated bacteria from healthy and bleaching *Acropora* corals of Blitar Waters, East Java, Indonesia

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Abstract. Global warming leads to high coral bleaching phenomena in marine ecosystems. The bleach condition of corals can cause a disease that is enhanced by opportunistic or pathogenic microorganisms. This research aimed to investigate the antagonistic interactions between healthy and bleached coral-associated bacteria. Isolation of coral-associated bacteria was carried out using the spread plate method in half-strength Zobell 2216E medium. Antagonistic activity was observed using a double-layer method, with each layer of medium inoculated with healthy or bleached coral-associated bacteria. The interactions between healthy and bleached coral isolates were analyzed using correspondence analysis. Twenty healthy and 11 bleached coral isolates were found in the six coral samples. Approximately 14 bacterial isolates from healthy corals demonstrated antagonistic (inhibitory) activity against 11 bacterial isolates from bleaching coral samples, with isolate AcD.14 from bleached coral, inhibited by 57.14% of the active isolates from healthy corals. Correspondence analysis resulted in isolate AcD.16, a bleaching coral bacterium with sufficient pathogenicity to elicit an antagonistic response from healthy coral bacteria. This study showed that there is high potential for finding bio-control agents for coral diseases using their natural microbiomes from healthy corals.

1 Introduction

The coral reef ecosystem is a coastal ecosystem with high biodiversity at the macro- and micro-scales. *Acropora* is one of the dominant coral genera in Indonesian water. *Acropora* have a relatively fast growth rate of approximately 6-8cm per year [1]. The population of *Acropora* corals is decreasing rapidly, one of which is caused by changes in environmental conditions that cause stress to coral biota [2]. Alterations in the surrounding environment can disrupt the interaction between coral animals and photosynthetic symbionts, namely Symbiodinium algae, resulting in coral bleaching [3]. Coral bleaching events increase coral morbidity and mortality, causing changes in the composition of coral communities [4].

The microbes found in the structure of healthy coral holobionts (host corals and their associated biota) consist of microbial communities that have different functions [5]. The microbial community can help adjust the coral holobiont to environmental changes by rapidly restructuring its composition [6]. Changes in the microbial community structure of coral reefs

can result in coral bleaching events or diseases caused by the presence of opportunistic or pathogenic microbes [7]. Therefore, the microbial composition of healthy corals is more complex than that of bleached corals. Among all kinds of microbes, bacteria are the easiest to observe and indicators of coral health. Several methods, such as culture methods, can be used to study coral-associated bacteria. The application of bacterial culture methods is essential in identifying bacterial communities that are crucial for the emergence of coral resistance [7-8].

Bacteria-culture methods from the genera *Endozoicomonas* and *Pseudoalteromonas* are found in the corals *Acropora cytherea*, *Acropora hemprichii*, and *Acropora sp.* have the highest antagonistic potential against the coral pathogen *Vibrio coralliilyticus* [9]. Bacteria associated with healthy and bleached corals have antagonistic characteristics, in which coral reefs develop self-defense mechanisms against their environment or other microorganisms [10]. The antagonistic potential is produced by the activity of bacterial antagonism, which is an inhibitory mechanism from one type of bacteria by a substance made by a different kind of bacteria [11]. Opportunistic bacteria found in bleached corals have the potential to become pathogenic when corals experience stress. This research aims to study the antagonistic activity of associated bacteria from healthy and bleached *Acropora* corals to restore coral bleaching.

2 Materials and methods

2.1 Site observation and sample collection

Sampling was carried out on November 20, 2020, in a coastal area with a water depth of 10m, which was \pm 670m from the shoreline (coordinates: 8°19'11"S, 112°08'23"E). The community structure of coral reefs was observed using a modified Line Intercept Transect (LIT) method by extending a 50m transect parallel to the shoreline [12]. The data focused on the primary substrate category. Approximately six samples of hard coral were collected from the genus *Acropora*, consisting of three samples of healthy coral and three samples of bleaching coral with a length of 5-7cm using a chisel and hammer [13]. Samples were taken from different colonies within an area of 100m² parallel to the coastline [9]. The sample was then stored temporarily using zip plastic and then transferred into a 250ml HDPE bottle containing 50% sterile seawater and 50% of 97% glycerol, then placed in a coolbox to be taken to the ITK Microbiology Laboratory, FPIK-IPB, for laboratory analysis.

2.2 Bacteria isolation

The coral tissue was cleaned of dirt and one gram of coral tip was taken and crushed using a sterile mortar and pestle for dilution. The dilutions of 10^{-3} , 10^{-4} , and 10^{-5} each were taken 100 μ L using a micropipette and cultured in a petri dish containing 15–20ml of Zobell 2216E medium using the spread cup method. The samples were isolated in triplicate and then incubated at 27°C for 96h [14]. The morphology and pigmentation of the incubated bacterial colonies were recorded. The different colonies were then inoculated using the quadrant scratch method on a Petri dish containing Zobell 2216E medium for purification. After obtaining pure isolates, colonies were cultured on tilted agar to proceed to the Gram staining stage. The Gram staining stage was carried out by taking one loop of cultured bacteria and then fixing it dry with Bunsen flame. The Gram staining stage utilizes the primary dye, crystal violet, and the counter dye, safranin. The bacterial cultures that underwent staining were then observed under a microscope at a magnification of 1000x.

2.3 Antagonistic assay

The antagonistic activity of coral-associative bacteria was tested using the double-layer method [15]. Healthy coral bacterial isolates were dotted on the surface of Zobell 2216E agar medium and incubated for 96h at 27°C. Around the same time, bleached coral bacterial isolates were cultured in liquid Zobell medium and incubated in a shaker for 96h. Subsequently, 100µL of bleached coral bacteria was poured on top of the healthy coral bacteria and mixed with 10ml of Zobell 2216E agar medium until homogenization. The plates were incubated for 96h at 27°C. The positive control used was 100ppm Tetracycline HCL to be tested on bleaching coral bacteria. Antagonistic activity between healthy coral bacterial isolates and bleaching corals was indicated by the formation of an inhibition zone (clear zone) around the healthy coral bacterial dots. The inhibition zone formed from the results of antagonist activity was photographed, and the diameter of the inhibition zone was measured using ImageJ software.

2.4 Data analysis

The data were analyzed by correspondence analysis (Correspondence Analysis/CA) using XLStat 2022 software and Microsoft Excel 2013. Correspondence analysis is a technique that allows multidimensional representations between rows and columns of tables with a two-way contingency [16].

3 Results and discussion

3.1 Life-coral abundance

One of the categories observed while examining the structure of coral reef ecosystems is the life coral category. The coral reefs found in the study area formed large patches at depths of 7-15m depth. In the 100m² observation area, five types of hard coral life forms were found: Coral Encrusting (CE), *Acropora* Coral Branching (ACB), *Acropora* Coral Digitate (ACD), Coral Foliose (CF), and Coral Branching (CB). The abundance of coral lifeforms types found at this location is shown in **Figure. 1**.

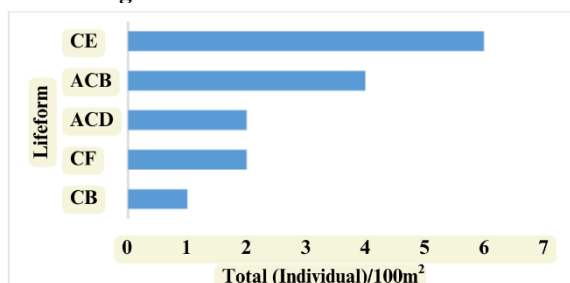


Fig. 1. Life coral abundance found at the study site.

The ACB life form has a branched shape similar to that of a tree branch and belongs to the *Acropora* coral life form. *Acropora* is a type of coral that grows faster and dominates shallow waters [17]. In addition, many studies have shown that *Acropora* is the coral that most often experiences bleaching because it is not resistant (susceptible) to changes. *Acropora* corals with the ACB life form were chosen as samples in this study because of their considerable abundance at the study site. Considering the ability of *Acropora* to grow faster

than other coral genera, the sustainability of coral stocks in nature was taken into consideration during the sampling process.

3.2 Bacteria macroscopic and microscopic characteristic

A total of 31 bacterial isolates were obtained, consisting of 20 healthy coral isolates and 11 bleached coral isolates. The coral holobiont is a complex unit that includes numerous related species of bacteria. Bacterial isolates obtained from healthy coral samples were more significant than coral bacterial isolates that experienced bleaching because corals that experienced bleaching were a product of changes in the coral microbial community, which caused corals to lose bacteria that could protect themselves [18]. The colony and cell characteristics of all the bacterial isolates are presented in **Figure 2**.

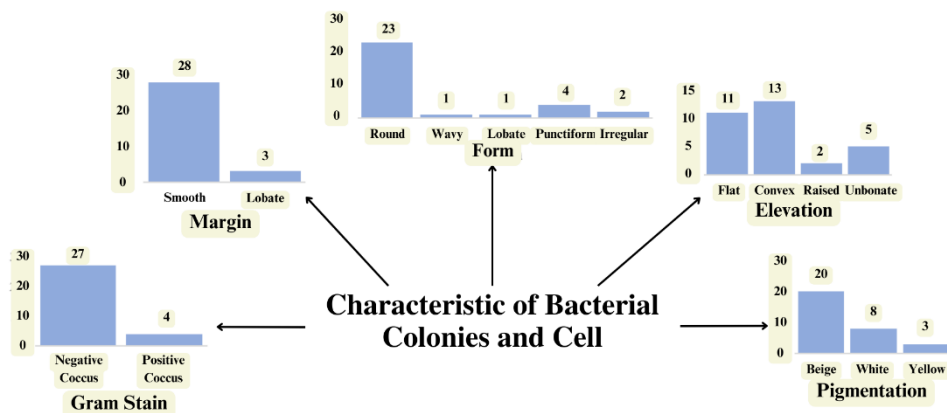


Fig. 2. Characteristics of bacterial colonies and cells.

The observation of the macroscopic characteristics of bacteria were observed by observing the edges, shape, elevation, and pigmentation, whereas microscopic observations were carried out by observing the gram and shape of the cells. Macroscopic observations showed that smooth edges consisted of 28 isolates (90.32%). Round shapes dominated colony forms, with 23 isolates (73.19%). Colony elevation was dominated by convex elevation with 13 isolates (41.93%). Colony pigmentation was dominated by beige or non-pigmented coloration in 20 isolates (64.51%).

Gram staining was performed to determine the bacterial forms and features. The results showed that 27 isolates were gram-negative with a coccus shape. Gram-negative bacteria are commonly found among scleractinian coral-associated bacteria. Bacteria with a coccus cell form that bonds with each other to create a strong (solid) surface because it has a slimy material so that the cells can be interconnected [19]. This allows the bacteria to form a surface layer and live in symbiosis [20].

3.3 Antagonistic activity

The results of the antagonist test showed that 14 healthy coral isolates had antagonistic activity against 8 bleaching coral isolates. The criteria for antagonistic activity based on the size of the inhibition zone are divided into four categories: weak activity with an inhibition zone size of ≤ 5 mm, moderate activity with an inhibition zone size of 5-10mm, strong activity with an inhibition zone size of 11-20mm, and very strong activity with an inhibition zone size of ≥ 20 mm [21].

A comparison of antagonistic activity against antibiotic compounds (tetracycline) was performed as a positive control. Tetracyclines are broad-spectrum antibiotics with activity against Gram-positive and Gram-negative microorganism infections [22]. This substance inhibits bacterial protein synthesis in the ribosomes [23]. The inhibition zone of tetracycline antibiotics with a diameter of 11 mm is resistant, an inhibition zone with a diameter of 12-14mm is intermediate, and an inhibition zone with a diameter >15mm is sensitive [24]. The results of the diameter measurements of the inhibition zones are presented in **Table 1**.

Table 1. Antagonistic assay of healthy coral isolates against bleaching coral isolates after three days of isolation, obtaining a wide range of inhibition zone sizes.

Isolate Code	Inhibition Zone Size (mm ± SD)							
	AcD.1	AcD.4	AcD.5	AcD.7	AcD.9	AcD.11	AcD.14	AcD.16
AcS.3	-	-	-	-	-	12.82 ± 0.42	-	-
AcS.6	-	-	-	-	-	-	5.87 ± 0.13	-
AcS.8	14.03 ± 0.43	-	-	-	-	-	-	-
AcS.10	17.45 ± 0.21	-	-	-	2.94 ± 0.52	-	4.95 ± 0.15	-
AcS.11	19.78 ± 0.13	19.05 ± 0.42	-	-	14.28 ± 0.49	-	18.78 ± 0.32	-
AcS.15	13.81 ± 0.23	-	-	-	2.22 ± 0.13	-	-	-
AcS.16	-	-	-	-	4.3 ± 0.68	-	5.38 ± 0.04	29.18 ± 0.24
AcS.18	-	-	8.04 ± 0.10	-	-	10.94 ± 0.55	-	8.82 ± 0.17
AcS.19	-	-	-	-	4.2 ± 0.81	-	6.85 ± 0.66	-
AcS.20	-	-	-	-	4.7 ± 0.17	-	4.50 ± 0.14	-
AcS.22	-	-	11.52 ± 0.12	-	-	12.38 ± 0.29	10.14 ± 0.07	8.6 ± 0.14
AcS.25	-	-	-	-	-	-	-	36.59 ± 0.23
AcS.26	-	17.65 ± 0.30	-	-	-	-	-	-
AcS.27	-	-	-	11.66 ± 0.63	-	-	26.27 ± 0.19	-
Positive Control	0.67 ± 0.21	0.72 ± 0.11	0.48 ± 0.03	0.69 ± 0.10	0.55 ± 0.05	1.51 ± 0.31	0.75 ± 0.13	0.63 ± 0.24

The inhibition zone formed by the antagonistic activity isolates was very diverse, ranging from 2.22 mm to 36.59 mm. There were seven potential antagonists with weak activity, eight potential antagonists with moderate activity, twelve potential antagonists with strong activity, and three potential antagonists with very strong activity. Isolate code AcS.25 had antagonistic activity against isolate AcD.16, with the largest inhibition zone area of 36.59 ± 0.23 mm. In contrast, the isolate AcS.15 inhibits isolate AcD.9 which had the smallest inhibition zone area of 2.22 ± 0.13 mm. The antagonistic ability of the test bacteria increased as the inhibition zone grew larger [25].

Naturally, the microorganisms associated with corals in these healthy/normal conditions are resistant to pathogenic microorganisms; however, when corals experience coral bleaching

events, the loss of potential microorganisms increases the growth rate of bacteria with the potential to become pathogens, causing coral hosts to become more susceptible to pathogens [26-27]. It is reasonable to assume that coral isolates that underwent bleaching with the code AcD.14 have the ability to cause pathogenicity or opportunistic bacteria with the potential to dominate because that it was the most responsive bleaching coral isolate by healthy coral isolates.

Overall, the inhibitory activity demonstrated a difference between the antagonist test (healthy corals on bleached corals) and the positive control (tetracycline on bleached corals). In the positive control, the diameter of the inhibition zone was relatively small, with the largest inhibition zone being 1.51 ± 0.31 AcD.11. Based on the assumption that bleached coral bacteria have a low sensitivity to tetracycline antibiotic compounds, the results show that these bacteria are more resistant.

3.4 Correspondence analysis

Correspondence analysis is a technique that makes it possible to find multidimensional representations between rows and columns in a two-way contingency table [16]. Correspondence analysis aims to determine the relationship or closeness of two variables [28]. The mapping results showed the best dimensions for presenting data from the variables of healthy coral isolates and coral isolates that had undergone bleaching, as shown in **Figure 3**.

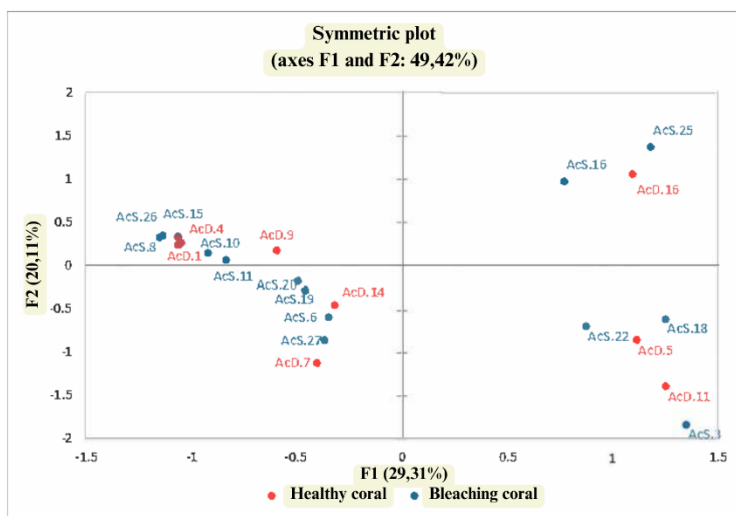


Fig. 3. Correspondent analysis (CA) results between healthy and bleached coral isolates.

The cumulative percentage shown in the corresponding analysis results was 49.42%, indicating that the mapping quality can explain 49.42% of the variability of the actual data. The results from the squared cosine variable in cluster F1 show that isolates AcS.18 and AcS.22 have cosine squared values of 0.61 and 0.46, respectively, indicating activity against isolate AcD.16, which has a cosine squared value of 0.51. This shows that AcS.22 isolates were the most effective at inhibiting bleaching among healthy coral isolates. Isolate AcS.22 inhibits isolate AcD.16 and has a high cosine squared value of 45%. Additionally, based on the biplot interpretation in Figure 3, we can understand the correlation between healthy and bleached isolates from each cluster, such as from cluster 1. Isolate AcD.16 has a closer coordinate to AcS.25 than AcS.16, which makes AcS.25 have a more immediate correlation with inhibiting AcD.16.

These results demonstrate that the AcD.16 isolate is a bleaching coral bacterium with sufficient pathogenicity to elicit an antagonistic response from healthy coral bacteria. As evidenced by the mechanism of antibacterial compound production, bacteria associated with corals have the ability to protect corals against potential pathogens [29] by stimulating protective mechanisms to compete for nutrients or space. Moreover, *Endoizoicomonas* strains, which are linked to *Acropora* corals, have been identified to have the potential to produce antimicrobial compounds [30]. Therefore, the antagonistic activity of the associative bacteria in the healthy coral samples used in this study may play a role in producing antibacterial compounds to protect the coral host. Therefore, the results of this study can help better understand microorganism interactions between healthy and bleached corals, which is essential for contributing to the resilience of coral hosts.

4 Conclusion

The microbial community on coral hosts plays a crucial role in protecting coral hosts. Through rapid restructuring of microbial community composition, coral holobionts can adapt to environmental changes. Unfortunately, using the spread plate method, the microbial structure of bleached coral found in this study has lost some major bacteria that play a role in protecting coral hosts, as evidenced by the identification of 20 healthy coral isolates and 11 bleached coral isolates. Healthy coral samples were found to have more associated bacteria than the bleached corals. A total of 12 antagonistic activities with a strong spectrum were shown by 14 healthy coral isolates against eight bleaching coral isolates, with an inhibitory zone range of 11.52 ± 0.12 mm to 19.78 ± 0.13 mm and aided with the correlation analysis results, where AcD.16 was found to have a strong correlation with AcS.22 by the value of cosine squared and with AcS.25 by the biplot coordinate proves that the healthy coral bacteria found in this study may play a role in producing antibacterial compounds to protect the coral host against potential pathogens. This study has also shown that in the coral holobiont, there are bacteria that have the ability to form self-defense mechanisms that are useful in maintaining the resistance and resilience of coral hosts in nature.

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Author DSA contributions included performing site observation, collected samples, laboratory analysis, data analysis, and wrote the paper. Author MSI contributed as the corresponding author, responsible for the entire research, developing the research ideas, conceived the analysis, designed the analysis, directed the methodology, and provided an evaluation of the discussion. Author NC was responsible for the whole research, developing research ideas, directing the methodology, and providing an evaluation of the discussion.

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