



## The bioprospecting of endophytic fungi in mangroves as natural anti-*Vibrio parahaemolyticus*

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### ABSTRACT

Vibriosis caused by *Vibrio*, such as *V. parahaemolyticus*, is one of several issues in shrimp farming. Antibiotic misuse in disease controlling is suspected of producing environmental contamination resistance of microbes, and rejecting the products. One of the efforts that can be made to overcome vibriosis is by utilizing endophytic fungi in mangroves. This is due to the similarity of bioactive compounds produced by mangroves and endophytic microorganisms. This study aimed to analyze the bioprospection of endophytic fungi as anti-*V. parahaemolyticus* at the Lampung Mangrove Center (LMC). The method used in the study was exploratory by isolating endophytic fungi from the aerial roots and leaves of mangrove plants that predominantly grow in LMC for further bioactivity screening process against *V. parahaemolyticus*. This study employs an exploratory approach with descriptive analysis. Based on the antibacterial activity test using the agar plug method, 9 out of 76 fungal isolates showed the ability to inhibit the growth of *V. parahaemolyticus*, namely isolates coded L-A1-MD1, L-A1-MD2, L-A1-MD3, L-A2-MA4, L-A2-MA5, L-A2-MA6, L-B1-MD18, L-P4-MA47, and L-A6-MA79. All isolates of endophytic fungi that had bactericidal activity against *V. parahaemolyticus* predominantly come from the *Avicennia marina* mangrove.

**Keywords:** Antibacterial, Bioprospecting, Endophytic Fungi, Lampung Mangrove Center, *Vibrio parahaemolyticus*



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### 1. Introduction

One of the major brackish water aquaculture products with significant economic value on domestic and international markets is white shrimp (*Litopenaeus vannamei*), which Indonesia produces 65% annually [1]. The growth of shrimp farming is frequently hampered by outbreaks of bacterial illnesses like vibriosis [2-4]. *V. parahaemolyticus* is known to be a pathogen that causes epizootic disease. It is an opportunistic bacteria that can cause disease in shrimp when under stressful conditions [5]. According to [6], the pathogenicity of *V. parahaemolyticus* is the presence of virulent genes in the form of TLH and TDH, which cause red blood cell lysis and anemia.

In intensive culture methods, the pathogenic agent that causes vibriosis can spread quickly and cause up to 85% mortality [7–8]. Research has shown that *L. vannamei*, *Penaeus monodon*, and *P. chinensis* are

susceptible to Early Mortality Syndrome (EMS) caused by bacteria in the genus *Vibrio* [9–10]. Commonly referred to as Acute Hepatopancreatic Necrosis Disease (AHPND), this condition can cause up to 74% mortality in China, Vietnam, Thailand, and Malaysia within 30 days of culture.

Antibiotics can lead to environmental contamination and resistance, according to [11]. Increasing resistance challenges aquaculture operations since it can significantly impair treatment [12]. Mangroves are one plentiful natural resource that has not been used to its full potential. Utilization of mangroves in shrimp health as an environmental service [13–14]. Many mangroves have bactericide compounds that can prevent shrimp from contracting vibriosis, according to [15]. Alkaloids, saponins, tannins, flavonoids, triterpenoids, and steroids are secondary metabolite compounds found in nearly every part of mangroves. Antibacterial activity tests can be carried out directly using parts of mangroves, but this exploits natural materials [16].

Mangroves must be used wisely for sustainability, so the solution is to use microorganisms associated with mangroves. Bioactive compounds can still be obtained from recombination between genetic microorganisms and their hosts, such as endophytic fungi in mangroves [17]. This is by [18] that the endophytic fungus *Pestalotiopsis* sp. isolated from the mangrove *Rhizophora* sp. contains secondary metabolite compounds such as glycol hydrolase and lignolytic enzymes. The glycol hydrolase enzyme plays a role in inhibiting bacterial peptidoglycan synthesis. Lignolytic enzymes can oxidize bioactive compounds such as phenol. Therefore, this research was carried out to achieve two interests simultaneously i.e utility value and sustainability. Management of disease in vaname shrimp culture in an environmentally friendly manner, without exploiting mangroves as a stock of natural germplasm. This research aims to analyze the bioprospection of endophytic fungi in mangroves as a natural anti-*V. parahaemolyticus* that can be used in vaname shrimp culture.

## 2. Method

An exploratory study using descriptive analysis was employed as the methodology. At the Lampung Mangrove Center, University of Lampung, Margasari Village, Labuhan Maringgai District, East Lampung Regency, mangroves were collected from 12 sampling points. The zoning of the mangrove area, which is quite dense, still near the sea, and where tides and light intensity are still conceivable, is used to determine the sampling points. Geographic Information System (GIS) analysis results using remote sensing satellite imagery can provide information to determine sampling points. This is also to determine the species composition and structure of mangrove vegetation at the sampling location. A map of the Lampung Mangrove Center area is presented in Figure 1.

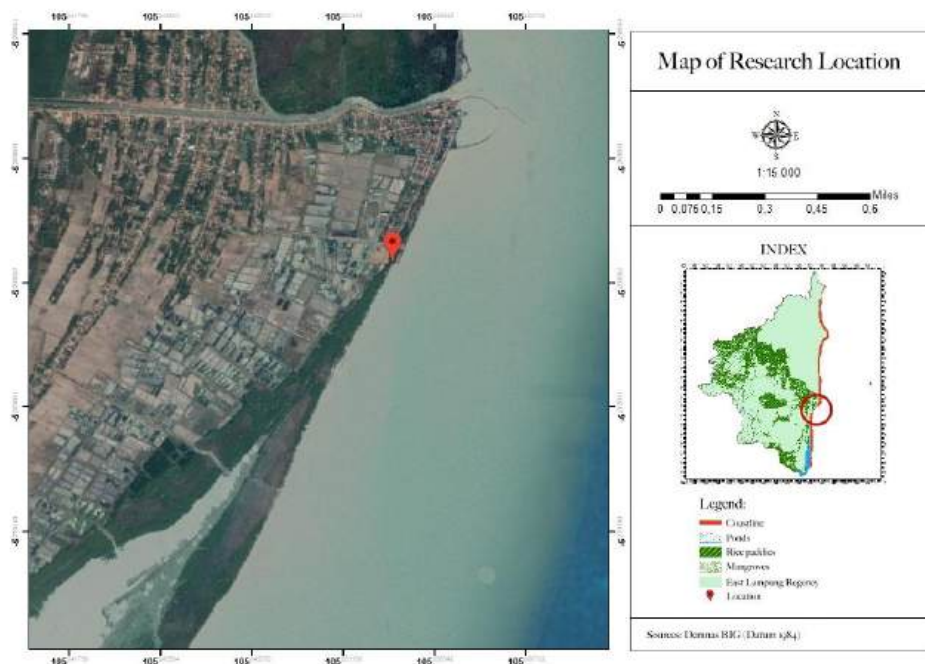


Figure 1. Map of sampling locations

The sampling method used is purposive sampling, namely mangrove samples taken based on mangrove species potentially containing endophytic fungi as antibacterials. The species of mangroves used include *Avicennia marina*, *Rhizophora apiculata*, *Acanthus ilicifolius*, *Nypha fruticans*, *Rhizophora mucronata*, and *Sonneratia caseolaris*. The isolated parts of the mangrove are the aerial roots and leaves [19-20]. Aerial roots are roots that are at the top and are not exposed to seawater or substrate, while the isolated parts of the leaves are the tips and veins of the leaves. Endophytic fungi originating from mangroves were isolated by washing the sample pieces with aquadest, spraying them with 70% alcohol, and then drying them in the air. The sample pieces are placed in a sterile plastic container and stored in a cool box. Mangrove samples must be immediately isolated into growth media namely Potato Dextrose Agar (PDA) before 24 hours to avoid contamination [21]. Sterilize glassware using soap, then rinse with water, dry, and wrap in paper and heat-resistant plastic. Glassware and materials such as growth media were sterilized in an autoclave at 121 °C and 1 atm pressure for 20 minutes. Stainless ware is sterilized using 70% alcohol and then burned over a bunsen fire just before use. The laboratory test process is carried out in laminar airflow and sterilized with UV for 30 minutes before it is ready for use.

To isolate mangrove endophytic fungi, 2x2 cm sample pieces were placed on PDA media and incubated for 5x24 hours at 25 °C. The growing fungus is purified into new PDA media using its morphological characteristics to obtain pure fungal isolates [22]. Characteristics of fungal isolates include colony color, colony texture, hyphae type, concentration lines, and growth zone lines. Fungal isolates with growth diameters of less than 1 cm, 1-2 cm, 2-3 cm, 4 cm, and >5 cm throughout the ideal incubation period of 7 days fall into the following growth categories: Very Slow, Slow, Medium, Fast, and Very Fast. This study employed the agar plug diffusion method to screen for antibacterial activity [23]. Inoculation of pathogenic bacteria in Trypticase Soy Broth (TSB) media with 2% NaCl. Incubation process for pathogenic bacteria for 24 hours at 37 °C. The bacterial density used refers to the 0.5 McFarland standard ( $1.5 \times 10^8$  CFU/ml). Bacteria were taken as much as 1% of the media volume to be grown on Trypticase Soy Agar (TSA) media using a sterile cotton swab. The incubation process for pathogenic bacteria is for 24 hours at a temperature of 37 °C [24]. Agar plugs from endophytic fungi are made by molding the top layer of pure fungal isolates that grow on PDA media into small spheres with a diameter of 6 mm using a sterile needle or blue-tip micropipette. The agar plug was placed upside down so that the fungal isolate could face the TSA medium directly, which had been previously smeared with *V. parahaemolyticus* bacteria. The clear zone formed around the endophytic fungal plug was measured for 2x24 hours [25].

### 3. Results and Discussion

#### 3.1. Isolation and Purification of Endophytic Fungi in Mangroves

Based on the sampling process at 12 points, 6 mangrove species were isolated. The isolation results showed 31 isolates of endophytic fungi in mangroves. Purification of endophytic fungi is the next step, yielding as much as 76 pure fungal isolates. Figure 2 displays the endophytic fungal purification findings for each species and part of the mangroves.

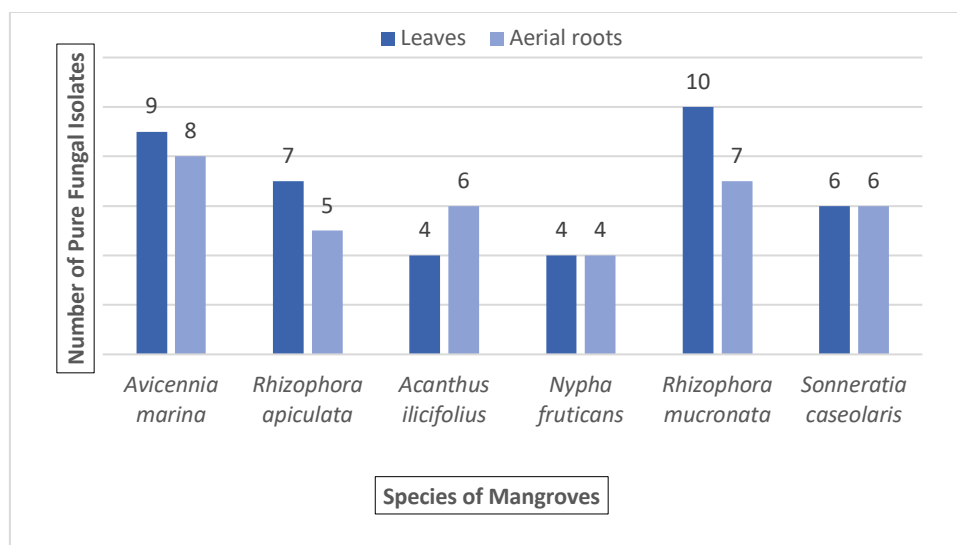


Figure 2. Results of Purification of Endophytic Fungi in Mangroves

The purification results will be utilized for the anti-*V. parahaemolyticus* activity screening test. Color, growth, and the presence or lack of filaments and spores are the traits used to purify fungi. Table 1 lists the morphological characteristics of the endophytic fungus isolates found in the Lampung Mangrove Center's mangroves.

Table 1. Characteristics of Endophytic Fungal Isolates in Mangroves at the Lampung Mangrove Center

Color	Growth	Structure
White (39 isolates)	Very Slow (19 isolates)	Filamentous (74 isolates)
Green (27 isolates)	Slow (9 isolates)	Spore (11 isolates)
Pink (6 isolates)	Medium (0 isolates)	
Black (4 isolates)	Fast (36 isolates)	
	Very Fast (12 isolates)	

Most fungal isolates are white, have a filamentous shape, grow quickly, and don't produce spores, based on the acquired pure isolates. Every group of fungi has unique traits, such as shape, structure, and life cycle in the environment, according to [26]. One crucial feature for the taxonomic identification of fungi is the shape of the spores and filaments.

### 3.2. Anti-*V. parahaemolyticus* Activity Screening Test

The agar plug method was used to screen for anti-*V. parahaemolyticus* activity in 76 endophytic fungal isolates; nine of these isolates formed a clear zone. The clear zone formed can be divided into 2 types: cidal and static. Metabolite compounds that eliminate pathogenic bacteria cause the cidal zone to become permanently clear. The antibacterial activity that creates the static zone is limited to preventing the growth of pathogenic bacteria. On day two, the static zone's antibacterial activity will decrease, reducing the clear zone's diameter. According to [27], bactericidal activity is defined by a persistently clean zone where bacterial growth is inhibited. According to [28], bacteriostatic action just prevents bacterial growth, which causes the clear zone's diameter to shrink gradually.

Endophytic fungal isolates with ability to combat *V. parahaemolyticus* include L-A1-MD1, L-A1-MD2, L-P4-MA47, and L-A6-MA79. Isolates with the codes L-A1-MD3, L-A2-MA4, L-A2-MA5, L-A2-MA6, and L-B1-MD18 can inhibit the growth activity of *V. parahaemolyticus*. Results of anti-*V. parahaemolyticus* activity screening test is presented in Table 2.

Table 2. Results of Anti-*V. parahaemolyticus* Activity Screening Test

No.	Fungal Isolate	Anti- <i>V. parahaemolyticus</i> Activity	
		Day-1	Day-2
1.	L-A1-MD1	++	++
2.	L-A1-MD2	++	++
3.	L-A1-MD3	++	+
4.	L-A2-MA4	++	+
5.	L-A2-MA5	++	+
6.	L-A2-MA6	++	+
7.	L-B1-MD18	++	+
8.	L-P4-MA47	++	++
9.	L-A6-MA79	++	++

Description: ++ : cidal type clear zone; + : static type clear zone

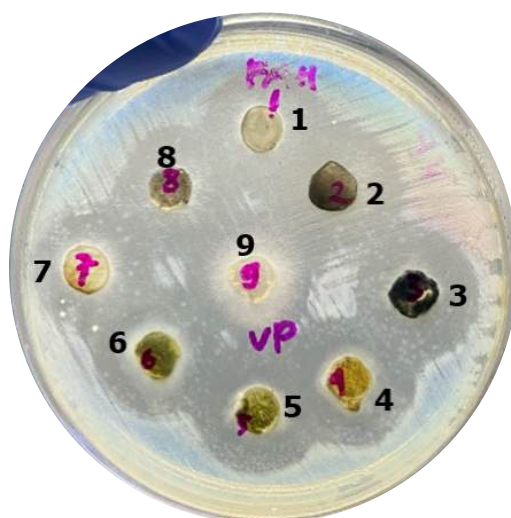
Based on the anti-*V. parahaemolyticus* activity screening test on 76 endophytic fungal isolates showed that 9 endophytic fungal isolates could form a clear zone with the diameter presented in Table 3.

Table 3. Clear Zone Measurement Results in Anti-*V. parahaemolyticus* Activity Screening Test

No.	Fungal Isolate	Clear Zone Diameter (mm)
1.	L-A1-MD1	19.70
2.	L-A1-MD2	18.98
3.	L-A1-MD3	13.96
4.	L-A2-MA4	13.44
5.	L-A2-MA5	13.57
6.	L-A2-MA6	13.84
7.	L-B1-MD18	13.60
8.	L-P4-MA47	16.57
9.	L-A6-MA79	16.23

The diameter of the inhibition zone, measured in millimeters (mm), is used to categorize an extract's capacity to stop bacteria growth. Inhibition zones are classified as Mild if their diameter is less than 5 mm, Moderate if their diameter is between 5 and 10 mm, Strong if their diameter is between 10 and 20 mm, and Extremely Strong if their diameter is greater than 20 mm. [29-31].

The formation of a clear zone is thought to be the endophytic fungal isolates L-A1-MD1, L-A1-MD2, L-A1-MD3, L-A2-MA4, L-A2-MA5, L-A2-MA6, L-B1-MD18, L-P4-MA47, and L-A6-MA79 can produce secondary metabolite compounds. The clear zone formed on anti-*V. parahaemolyticus* screening test is a Strong category presented in Figure 3.

Figure 3. The Clear Zone Formed on Anti-*V. parahaemolyticus* Activity Screening Test

- Information: (1): clear zone formed by the endophytic fungus L-A1-MD1  
 (2): clear zone formed by the endophytic fungus L-A1-MD2  
 (3): clear zone formed by the endophytic fungus L-A1-MD3  
 (4): clear zone formed by the endophytic fungus L-A2-MA4  
 (5): clear zone formed by the endophytic fungus L-A2-MA5  
 (6): clear zone formed by the endophytic fungus L-A2-MA6  
 (7): clear zone formed by the endophytic fungus L-B1-MD18  
 (8): clear zone formed by the endophytic fungus L-P4-MA47  
 (9): clear zone formed by the endophytic fungus L-A6-MA79

The fungus's antibacterial activity is demonstrated by the development of a clear zone [32]. Natural compounds derived from marine fungi have the potential to be used pharmacologically to vaname shrimp culture, according to [33]. It has been suggested that using endophytic fungi in mangroves is one way to combat vibriosis [17]. Figure 4 shows the morphology of endophytic fungal isolates from mangroves could be anti-*V. parahaemolyticus*.



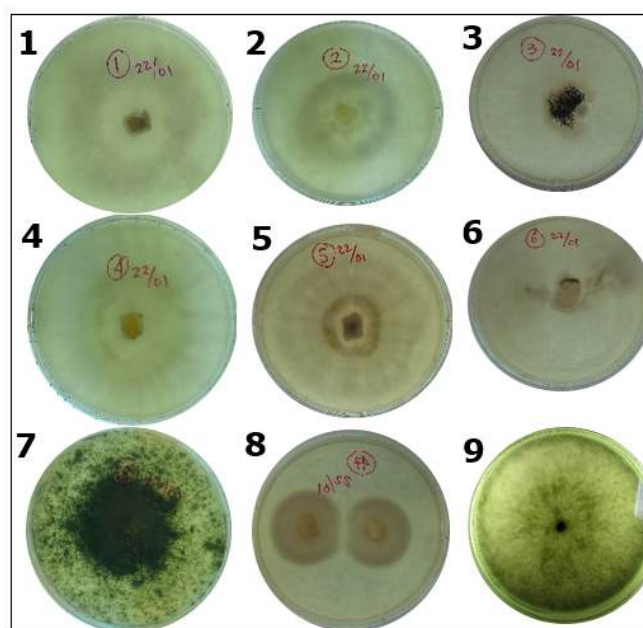


Figure 4. Endophytic Fungi Isolates in Mangroves with Potential Anti-*V. parahaemolyticus*

Information: (1): endophytic fungal isolate L-A1-MD1  
 (2): endophytic fungal isolate L-A1-MD2  
 (3): endophytic fungal isolate L-A1-MD3  
 (4): endophytic fungal isolate L-A2-MA4  
 (5): endophytic fungal isolate L-A2-MA5  
 (6): endophytic fungal isolate L-A2-MA6  
 (7): endophytic fungal isolate L-B1-MD18  
 (8): endophytic fungal isolate L-P4-MA47  
 (9): endophytic fungal isolate L-A6-MA79

Based on Figure 4, endophytic fungal isolates 1 and 2 have similar morphology, namely greenish white isolates, filamentous and fast-growing. Endophytic fungal isolates 4 and 5 have similar morphology i.e dark greenish white, filamentous, and grow very fast. Endophytic fungal isolates 3 and 6 have similar morphology, namely white and slightly black, filamentous, and grow very slowly. Endophytic fungal isolate number 7 has the morphology of a dark green isolate, filamentous, spore-forming, and fast-growing. Endophytic fungal isolate number 8 has a yellowish-white, filamentous isolate morphology and medium growth. Endophytic fungal isolate 9 has a dark green, filamentous isolate morphology and fast growth. Morphological observations on the nine pure fungal isolates can be used as supporting data in microscopically and molecularly identification.

Based on Figure 4, endophytic fungal isolates 1, 2, and 3 were isolated from *A. marina* mangrove leaves. Endophytic fungal isolates number 4, 5, 6, and 9 were isolated from the aerial roots of *A. marina* mangrove. According to [16], almost all parts of mangroves have secondary metabolite compounds as antibacterials. This is based on the statement [34] that endophytic fungi can produce compounds that their hosts also produce through genetic recombination. The endophytic fungus *Pseudopestalotiopsis* sp. mangroves have the same bioactive compounds as their hosts [35]. According to [36], they successfully isolated 2 species of endophytic fungi from the leaves, stems, and aerial roots of *A. marina* mangrove and even developed drugs for humans.

Based on Figure 4, endophytic fungal isolate number 7 was isolated from leaves of *R. apiculata* mangrove. Endophytic fungal isolate number 7 inhibited the growth of *V. parahaemolyticus* with a clear zone diameter of 13.60 mm. Based on research [38] and [46], it was found that the mangrove *Rhizophora* sp. can be used as an alternative treatment for diseases caused by bacteria of the genus *Vibrio*. *Rhizophora* sp. mangrove has antibacterial properties [37]. Based on previous research, extract of the endophytic fungus *Pseudopestalotiopsis* there from isolated aerial roots of *Rhizophora* sp. mangrove is used as a natural antibacterial. Endophytic fungi are found in tissues and are not harmful to their hosts [38].

Based on Figure 4, endophytic fungal isolate number 8, isolated from aerial roots of the *S. caseolaris* mangrove, was able to combat *V. parahaemolyticus* with a clear zone diameter of 16.57 mm. The endophytic

fungus *Pseudoestalotiopsis* sp. was isolated from the leaves of the mangrove *Sonneratia* sp. It is known to contain antibiotic compounds [39]. Metabolite compounds produced by endophytic fungi in mangroves include phenols, flavonoids, alkaloids, sterols, anthocyanins, and antioxidants [40-41]. This is by [42], which states that interactions between microorganisms and their hosts involve the transfer of genetic material. According to [43-44], the endophytic fungus *Trichoderma* sp. was also isolated from mangrove sediments, which have the potential to act as anti-*V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus*, and *E. coli*. [37] also stated that the extract from the endophytic fungus *Trichoderma harzianum* contains phenolic compounds.

The pathogenic ability of bacteria towards their hosts varies. The host's defense against pathogens influences this, as does the ability of pathogenic bacteria to produce toxins, enzymes, and plasmids, overcome host resistance, and increase reproduction speed [45]. Thermo Labile Haemolysin (TLH), Thermostable Direct Haemolysin (TDH), Thermostable Related Haemolysin (TRH), and adhesin genes control the production of thermolabile hemolysin, which is what makes *V. parahaemolyticus* pathogenic. TLH, TDH, TRH, and adhesin are virulence factors that cause disease from *V. parahaemolyticus* strains. The virulent gene produced by *V. parahaemolyticus* infects vaname shrimp through wounds in the exoskeleton and spreads through the hemolymph in the circulatory system. This gene produces hemolysin, which causes red blood cells to lyse, resulting in anemia and loss of body fluids. *V. parahaemolyticus* can be inactivated if heated to over 80 °C for 1 minute [6]. This is confirmed by [28], that saponins and flavonoids can interact with DNA in pathogenic bacteria, which disrupts bacterial cell metabolism. Alkaloids also reduce the permeability of bacterial cell walls and damage peptidoglycan; the cytoplasm will lyse so that the cells lack nutrition, then bacterial growth is inhibited, and then they die.

#### 4. Conclusion

Endophytic fungi isolated from *Avicennia marina* leaves and aerial roots have been shown to be bactericidal against *V. parahaemolyticus*. Meanwhile, endophytic fungal isolates originating from *Rhizophora apiculata* leaves inhibited the growth (bacteriostatic) of *V. parahaemolyticus*. Bioprospecting of endophytic fungi from these two species of mangroves can be used as a natural anti-*V. parahaemolyticus* in vaname shrimp culture.

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