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Short Communication

Isolation, Identification, and Pathogenicity of Potential Probiotics Isolated from Intestinal Coconut Crab (*Birgus latro Linnaeus*, 1767)

Muhammad Aris*[1], Tamrin Tamrin[1], Waode Munaeni[1], Sudirto Malan[1], Juharni Juharni[1], and Rusmawati Labenua[2]

[1]Department of Aquaculture, Faculty of Fisheries and Marine Science, Khairun University, Ternate City, North Maluku Province, 97719. Indonesia
[2]Department of Aquatic Resource Management, Faculty of Fisheries and Marine Science, Khairun University, Ternate City, North Maluku Province, 97719. Indonesia

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*) Corresponding author:
E-mail: amboasse100676@gmail.com

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Abstract

The supply of coconut crabs (*Birgus latro* Linnaeus, 1767) has been sourced from capture in the wild. Development efforts in aquaculture have succeeded in producing eggs. However, further development with larval maintenance failed because it was constrained by a low survival rate. This study aimed to identify the potential probiotic bacteria in coconut crabs. Coconut crab samples were collected from Moor Island, South Patani District, Central Halmahera Regency, North Maluku Province, Indonesia. The intestines of coconut crabs were taken as much as 1 g, then ground on a porcelain dish and added to Tri-Salt. The scouring liquid was taken in 0.1 ml and spread on a petri dish containing Nutrient Agar. The isolated used were marked with BL1, BL2, BL3, BL4, and BL5. Molecular identification was performed with sequencing and then analyzed using the Basic Local Alignment Search Tool (BLAST). The selection of isolates from the gastrointestinal tract of coconut crabs showed that the bacteria obtained were *Enterobacter tabaci* (BL1), *Enterobacter hormaechei* (BL2), *Bacillus horneckiae* (BL3), *Pseudomonas stutzeri* (BL4), and *Acinetobacter variabilis* (BL5). Further testing revealed that the bacteria *A. variabilis* (BL5) is a probiotic isolate in coconut crabs.

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1. Introduction

The coconut crab (*B. latro* Linnaeus, 1767) is the largest terrestrial arthropod in the world that lives near the coast. This species has a more active habit of foraging at night (Drew *et al*., 2010). The coconut crab is classified as a commodity that has a high economic value due to its high price and consumption, which emphasizes prestige (Widiyanti *et al*., 2015).

The supply of coconut crabs (*Birgus latro* Linnaeus, 1767) has been sourced from capture in the wild. This will result in a decline in the natural population. Coconut crabs have been classified as red-listed by the International Union for Conservation Network (IUCN) (Poupin *et al*., 2013). Various studies have been carried out on how to restore the population of these crabs, both from bioecological, reproductive, and genetic aspects, including efforts to develop them through aquaculture (Jefri *et al.,* 2020; Sulistiono *et al*., 2019; Serosero *et al*., 2020; Yorisue *et al*., 2020).

The expansion of coconut crabs into aquaculture has resulted in the successful production of eggs. However, further development with larval maintenance failed because it was constrained by low survival rate. Research conducted by Sugizaki *et al.* (2010) showed a decrease in the survival rate from the zoea phase to the megalopa phase, with the percentage of 100% decreasing to 71% and 82%, respectively. Different results were reported by Hamasaki *et al.* (2013), which revealed the occurrence of total mortality due to low energy which prevented growth.

Fish or crustaceans at the larval stage are highly dependent on the availability of indigenous feed or egg yolks, which serve as their primary energy source (Altaff, 2020). The high mortality of coconut crab larvae is thought to be related to a lack of egg yolks as well as their inability to utilize exogenous feed in the environment. One alternative solution to this problem is to increase the availability of exogenous gastrointestinal enzymes by utilizing bacteria from the gastrointestinal tract that have amylolytic, proteolytic, and lipolytic activity (Maas *et al*., 2021; Muttharasi *et al*., 2021).

Various studies have been carried out using probiotic bacteria from the gastrointestinal tract of crustacean species (Mohamad *et al*., 2020; Xu *et al*., 2021). Probiotic bacteria native to the gastrointestinal tract have a mutualistic symbiotic relationship with their host, which can increase feed utilization, growth, and cultivation immunity (Lee *et al*., 2021; Nimrat *et al*., 2021). In addition, these bacteria regulate the microbial environment in the intestine by suppressing the growth of pathogenic microorganisms (Chiu *et al*., 2021; Restrepo *et al*., 2021). Identification of potential probiotic bacteria in aquaculture systems is important (Ringø *et al*., 2020).

The identification of potential probiotic bacteria in coconut crab was not widely explored. Therefore, this study aims to identify potential probiotic bacteria isolated from the intestine of coconut crab. In addition, this research will be useful for the development of coconut crab for aquaculture.

2. Materials and Methods

2.1 Sample Collection

This study was conducted from March to December 2020. The Coconut crab samples used as sample material in this study were collected on Moor Island, South Patani District, Central Halmahera Regency, North Maluku Province, Indonesia ($0^0$10'52.09"N and $128^0$57'21.214"E). Coconut crab samples were within the size of 400–600 g and were caught at night. The fishing process was carried out using coconut as bait. At the time of catching, avoid shining the flashlight directly on the coconut crabs. Five test samples were placed in separate sacks and sent to the Wet Laboratory, Faculty of Fisheries and Marine Affairs, Khairun University.

2.2 Bacterial Isolation

Isolation was carried out at the System and Technology Laboratory, Aquaculture Study Program, Faculty of Fisheries and Marine Affairs, Khairun University (0^o47'26.34"S and 127^o23'3.264"E). The tools used to isolate bacteria are first sterilized. Each sample of Coconut Crab was dissected, and the intestines were taken as much as 1 g, then ground on a porcelain dish and added to Tri-Salt (Bio-Tech Pharmacal) solution. The scouring liquid was taken in 0.1 ml and spread on a petri dish containing Nutrient Agar (Merck, Germany). The isolated were marked with BL1, BL2, BL3, BL4, and BL5. The results of bacterial isolation were then scraped several times to obtain pure isolates. After pure isolates of bacteria were obtained, the colony type was evaluated and followed by molecular identification.

2.3 Molecular identification

Molecular identification was carried out using the 16-sRNA gene. Pure isolates were prepared in test tubes to be sent to the Brackish Water Cultivation Fisheries Research Institute and Fisheries Extension, Maros Regency, South Sulawesi. Furthermore, sequencing was carried out using the services of a Singaporean Research

Biolabs company. The results were then analyzed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI). The results of the analysis were then compared with similar bacteria deposited as in Sukenda *et al*. (2020). In addition, phylogenetic analysis with 1000 bootstraps was also performed using MEGA-X software, which compared the isolate with bacteria NR_024570.1 on NCBI data following Nuidate *et al*. (2021).

2.4 Pathogenicity Test

Pathogenicity tests were carried out at the Wet Laboratory, Faculty of Fisheries and Marine Affairs, Khairun University. The test animals used were *Litopenaeus vannamei* (0.10±0.00 g) certified free of pathogens from PT. Benur Kita, Barru Regency, South Sulawesi. This experiment used the statistical method of Completely Randomized Design (CRD) with a factorial pattern consisting of six treatments with three replications each and two groups. Treatments consisted of isolates BL1, BL2, BL3, BL4, BL5, and control without bacteria (with PBS). Groups consisted of fresh and seawater salinities of 0 mg L^{-1} 29 mg L^{-1}, respectively. The acclimatization of test animals from seawater to freshwater was done by gradual dilution from 29 mg L^{-1} to 0 mg L^{-1}.

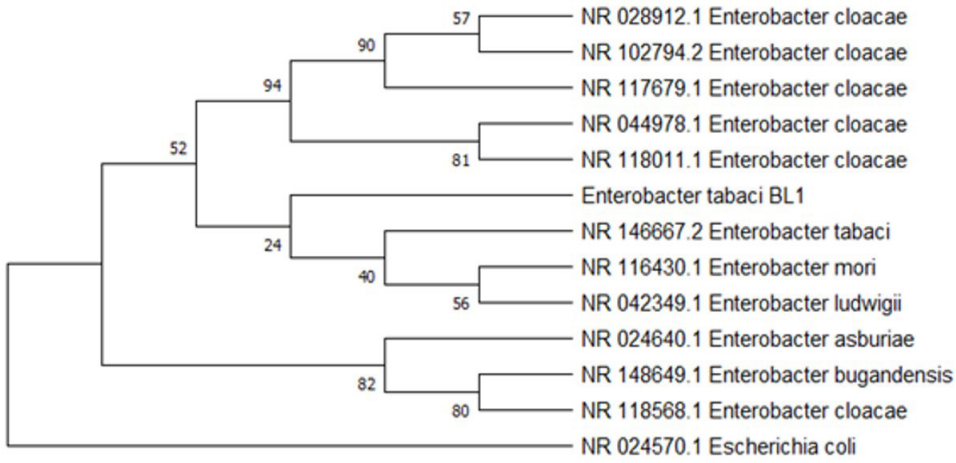

Figure 1. Phylogenic isolates of *E. tabaci* strain BL1.

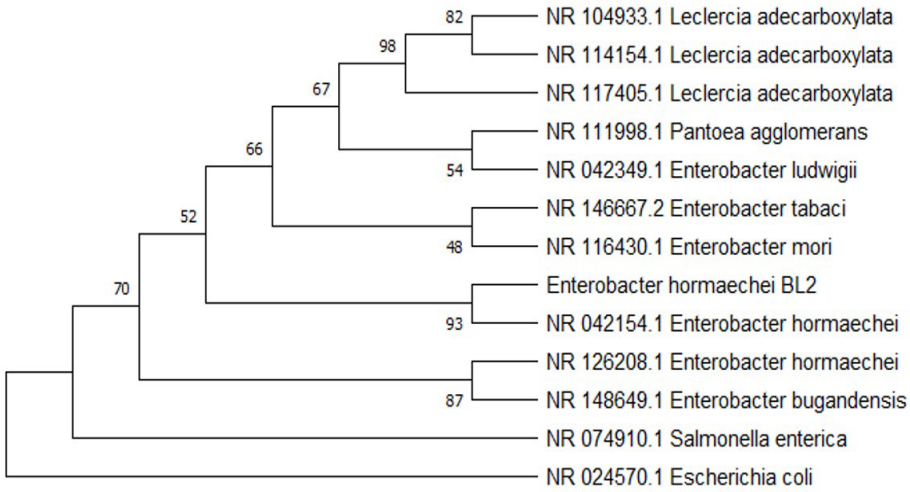

Figure 2. Phylogenic isolates of *E. hormaechei* strain BL2

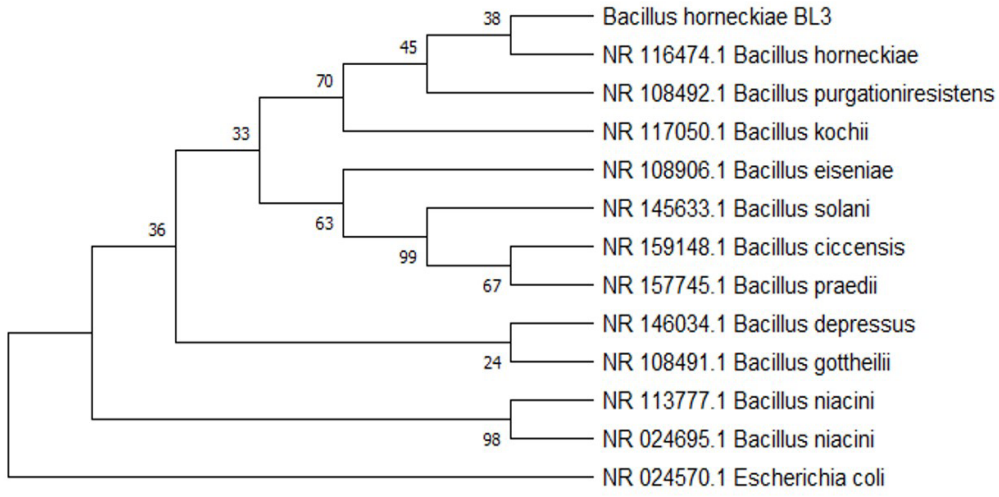

Figure 3. Phylogenic isolates of *B. horneckiae* strain BL3.

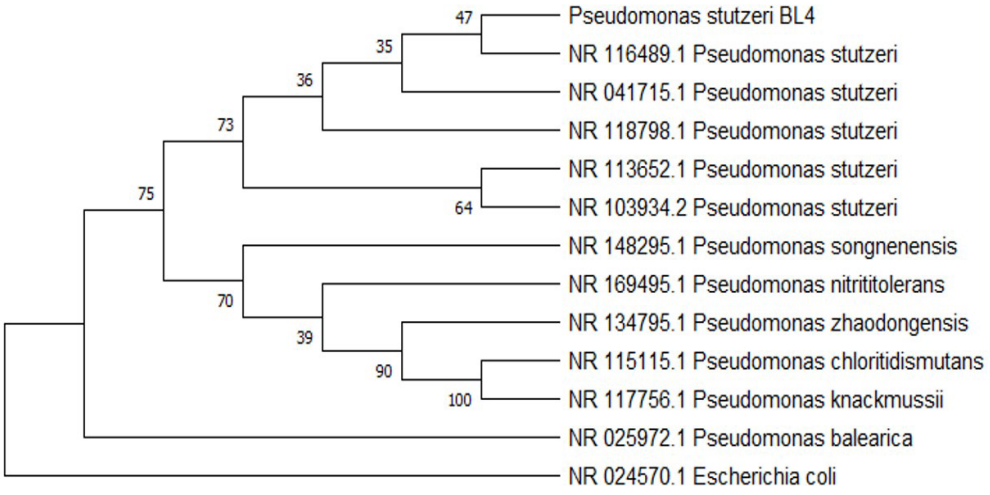

Figure 4. Phylogenic isolates of *P. stutzeri* strain BL4.

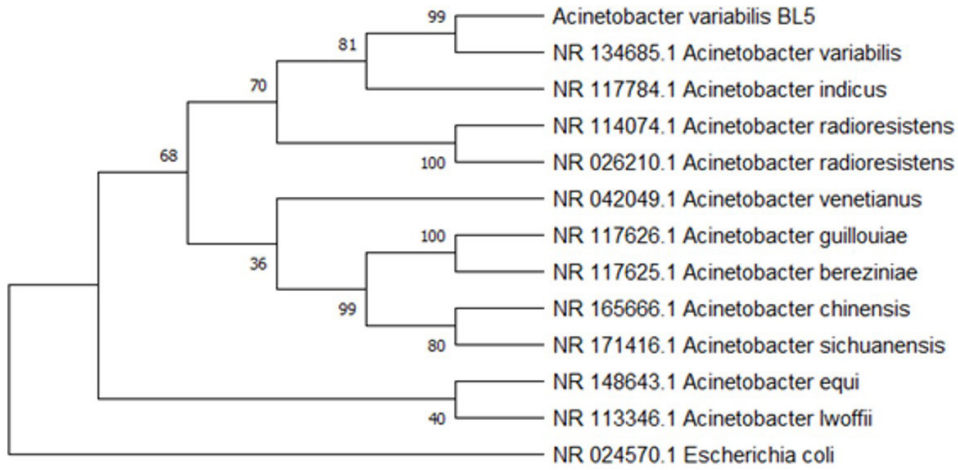

Figure 5. Phylogenic isolates of *A. variabilis* strain BL5.

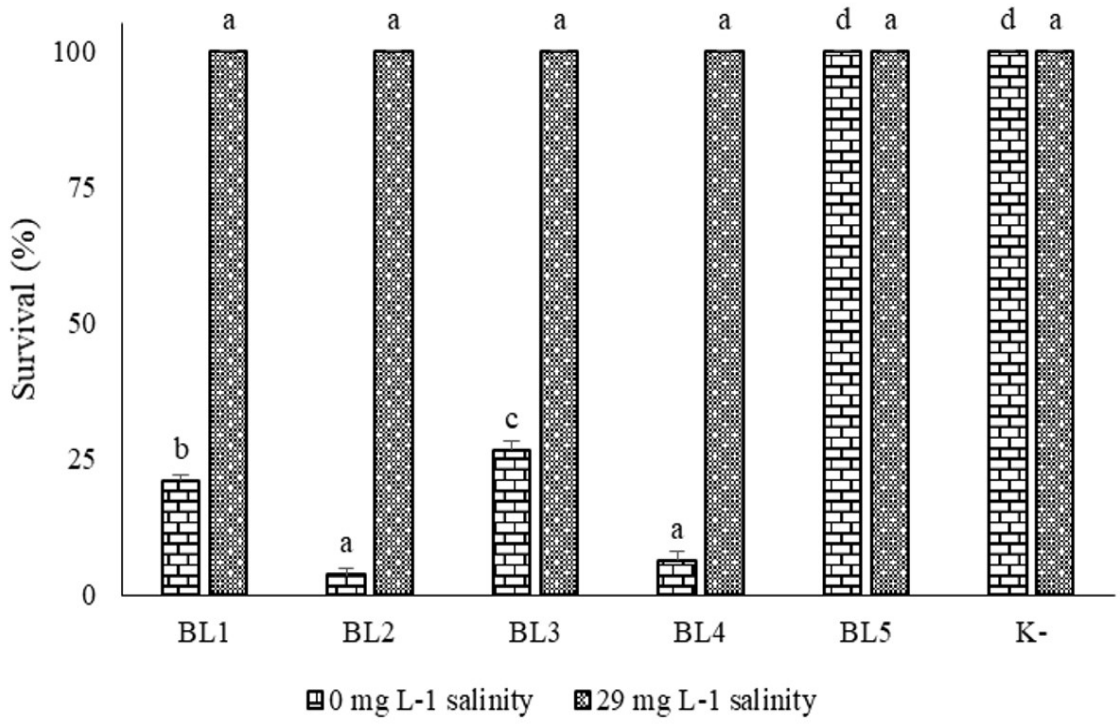

Figure 6. Survival rates of test animals in each treatment. *E. tabaci* strain BL1 (A), *E. hormaechei* strain BL2 (B), *B. horneckiae* strain BL3 (C), *P. stutzeri* strain BL4 (D), *A. variabilis* strain BL5(E) and negative control (K-). Different superscripts are significantly different ($P<0.05$).

Pathogenicity tests were conducted following He *et al*. (2021) with slight modifications. Each bacterial isolate was prepared with a concentration based on 10^6 CFU ml^{-1}. Isolates were first cultured on Nutrient Agar media (Merck, Germany) for 18 hours at 30°C, then inoculated into 150 ml of Nutrient Broth media (Merck, Germany) for 24 hours at 30°C. The culture was then centrifuged at 3500 rpm for five minutes. The separated supernatant was discarded, and the bacterial pellet was added to phosphate buffered saline (PBS: 8 g of NaCl, 1.5 g of Na_2HPO_4, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 1000 mL of distilled water), then homogenized with a vortex (this process was done twice). Furthermore, testing was carried out with an immersion system. The parameter observed was survival using the following methods as described by Ninwichian *et al.* (2022): SR (%) = (the existing number of shrimp at the end of the experiment/total number of shrimps at the beginning) x 100.

2.5 Analysis Data

The data were analyzed using Microsoft Excel 2010 and tested with analysis of variance (ANOVA), if there was a significant effect, Tukey's follow-up test was applied.

3. Results and Discussion

3.1 Results

3.1.1 Identification Bacteria

The results of molecular identification showed that the bacteria obtained from the intestinal tract of the coconut crab were *E. tabaci, E. hormaechei, B. horneckiae, P. stutzeri,* and *A. variabilis* (Table 1). Further, the phylogenetic analysis revealed that BL1, BL2, BL3, BL4, and BL5 isolates were similar to *E. tabaci* (Figure 1), *E. hormaechei* (Figure 2), *B. horneckiae* (Figure 3), *P. stutzeri* (Figure 4), and *A. variabilis* (Figure 5).

3.1.2 Pathogenicity Bacteria

The pathogenicity test results showed that there was no mortality in the seawater media test model (salinity 29 mg L-1) (Figure 6). However, the freshwater media test model (0 mg L-1 salinity) gave different results where the isolated *E. hormaechei* strain BL2 showed the highest level of pathogenicity compared to *E. tabaci* strain BL1, *B. horneckiae* strain BL3, and *P. stutzeri* strain BL4. Meanwhile, *A. variabilis* strain BL5 isolate showed non-pathogenic properties identical to the control treatment (PBS).

Table 1. Results of homology analysis of the 16-sRNA gene sequences of isolates

Gen Target	Isolate	Description	Accession number
16-sRNA	BL1	*Enterobacter tabaci* strain YIM Hb-3	NR_146667.2
		Enterobacter mori strain R18-2	NR_116430.1
		Enterobacter cloacae strain LMG 2683	NR_044978.1
		Enterobacter cloacae strain DSM 30054	NR_117679.1
		Enterobacter cloacae strain 279-56	NR_028912.1
	BL2	*Enterobacter hormaechei* strain 0992-77	NR_042154.1
		Enterobacter tabaci strain YIM Hb-3	NR_146667.2
		Enterobacter hormaechei strain 10-17	NR_126208.1
		Pantoea agglomerans strain JCM1236	NR_111998.1
		Enterobacter bugandensis strain 247BMC	NR_148649.1
	BL3	*Bacillus horneckiae* strain 1P01SC	NR_116474.1
		Bacillus kochii strain WCC 4582	NR_117050.1
		Bacillus purgationiresistens strain DS22	NR_108492.1
		Bacillus depressus strain BZ1	NR_146034.1
		Bacillus gottheilii strain WCC 4585	NR_108491.1
	BL4	*Pseudomonas stutzeri* strain CCUG 11256	NR_118798.1
		Pseudomonas stutzeri strain ATCC 17588	NR_041715.1
		Pseudomonas stutzeri strain NBRC 14165	NR_113652.1
		Pseudomonas songnenensis strain NEAU-ST5-5	NR_148295.1
		Pseudomonas stutzeri strain VKM B-975	NR_116489.1
	BL5	*Acinetobacter variabilis* strain NIPH 2171	NR_134685.1
		Acinetobacter guillouiae strain ATCC 11171	NR_117626.1
		Acinetobacter indicus strain A648	NR_117784.1
		Acinetobacter radioresistens strain NBRC 102413	NR_114074.1
		Acinetobacter equi strain 114	NR_148643.1

3.2 Discussion

Isolation of bacteria from the intestine of coconut crab resulted in four pathogenic isolates, namely *E. tabaci* strain BL1, *E. hormaechei* strain BL2, *B. Horneckiae* strain BL3, and *P. stutzeri* strain BL4. Meanwhile, one isolate, *A. variabilis* strain BL5, was found nonpathogenic after infection in test animals. Isolates BL1 and BL2 are major pathogenic bacteria in aquaculture. This pathogen was reported to attack the crustaceans *Procambarus clarkia* and *Macrobrachium rosenbergii* (Dong *et al*., 2020; Ma *et al*., 2020). The pathogenic bacterium *Enterobacter* is known to produce enterotoxins that attack internal organs and cause death in the host (Oliveira *et al*., 2017; Yang *et al*., 2017).

Isolate BL3 was a species of *Bacillus* bacteria that was disclosed as a pathogenic bacterium. *Bacillus* sp. is generally considered a probiotic bacterium and has been applied in aquaculture activities (Anokyewaa *et al*., 2021). However, several studies have also revealed something identical to this study that can cause the death of crustaceans *L. vannamei* and *Daphnia magna* (Sineva *et al*., 2009; Velmurugan *et al*., 2015). In addition, it was also disclosed as the causative agent of ice-ice in seaweed (Riyaz *et al*., 2021).

Isolate BL4 is a type of *Pseudomonas* bacteria that is the main pathogen in aquaculture activities. This bacterium is a disease-causing agent that attacks fish farming activities such as *O. niloticus* and crustacean aquaculture activities such as *M. rosenbergii* (Hindu *et al*., 2018; Osman *et al*., 2021). In addition, the pathogen *Pseudomonas* was revealed as the causative agent of ice-ice in *Kapphapycus alvarezii* seaweed which reduced its carrageenan quality (Riyaz *et al*., 2020).

Isolate BL5 is a type of *Acinetobacter* bacteria that is expressed as probiotic bacteria. The probiotic Acinetobacter in the intestines of *Seriola lalandi* fish is antagonistic by inhibiting the growth of the pathogenic bacterium *Vibrio* sp. (Ramírez *et al*., 2020). In addition, the use of *Acinetobacter* probiotic bacteria in *Clarias macrocephalus* could increase growth, survival, and immunity to *Aeromonas hydrophila* (Bunnoy *et al*., 2019).

The present study revealed that the bacteria *A. variabilis* strain BL5 is a potential probiotic in coconut crab. The application of this isolate was able to solve the problem of coconut crab larvae's low survival (Sugizaki *et al*., 2010; Hamasaki *et al*., 2013). Probiotic bacteria isolated from the host's gastrointestinal tract, in addition to maintaining survival rates, are also revealed to be able to improve growth, digestibility, and host immunity (Mohamad *et al*., 2020; Xu *et al*., 2021).

4. Conclusion

Isolates identified in the intestine of coconut crabs were *E. tabaci, E. hormaechei, B. horneckiae, P. stutzeri*, and *A. variabilis*. *E. tabaci*, *E. hormaechei*, *B. horneckiae*, and *P. stutzeri* have pathogenic characteristics. Meanwhile, *A. variabilis* is non-pathogenic. Further research needs to be done on the utilization of *A. variabilis* strain BL5 bacteria for the growth, digestibility, and immunity of coconut crabs.

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Authors' Contributions

The contribution of each author is as follows, MA, TT: Conceived and designed the analysis, verified the data, and wrote the original draft. MA, SM, RL: Conducted the research, collected the data, prepared the original draft. WM, JJ: Supervision, reviewed and edited the manuscript, and verified the data. All author discussed and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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