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Research Article

Anti-*Vibrio* Compounds from Digestive Symbiont Bacteria of Whiteleg Shrimp (*Litopenaeus vannamei*) from Traditional Ponds in Kaur, Bengkulu, Indonesia as Probiotic Candidates for Shrimp Aquaculture

Risky Hadi Wibowo[1,2,3]*, Reza Wahyuni[6], Iman Rusmana[4], Sipriyadi[1,2], Rahmad Lingga[5], Person Pesona Renta[7,8], Aceng Ruyani[9]

1) Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu, Kandang Limun, Bengkulu 38122, Indonesia
2) Master Study Program of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu, Kandang Limun, Bengkulu 38122, Indonesia
3) Research Centre of Sumatera Natural Products and Functional Materials, Universitas Bengkulu. Jl. W.R. Supratman, Bengkulu 38122, Bengkulu, Indonesia
4) Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Meranti, Bogor 16680, Indonesia
5) Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Bangka Belitung, Merawang, Bangka Belitung 33172, Indonesia
6) Graduate Student, Master Study Program of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu, Kandang Limun, Bengkulu 38122, Indonesia
7) Program of Marine Science, Faculty of Agriculture, Universitas Bengkulu. Jl. W.R. Supratman, Bengkulu 38371, Bengkulu, Indonesia
8) Department of Biotechnology and Bioindustry Sciences, National Cheng Kung University. Daxue Road No. 1, Tainan, Taiwan
9) Magister Program in Science Education, Bengkulu University, Jalan Raya Kandang Limun, Bengkulu 38371, Indonesia
* Corresponding author, email: riskyhadiwibowo80@gmail.com

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ABSTRACT

Kaur Regency is one of the areas with high potential for whiteleg shrimp pond cultivation, but it often faces shrimp deaths and crop failure due to infection with pathogenic *Vibrio* spp. and antibiotic resistance. One alternative preventive is to utilise potential symbiotic bacteria from the digestive tract that can produce anti-*Vibrio* and enzymatic activities. This study aimed to select potential digestive symbiont bacteria based on their anti-*Vibrio* ability through isolation, purification, identification, biochemical tests, and anti-*Vibrio* tests. Candidates showing activity were further tested for toxicity, hemolysin, effectiveness, enzymatic, and molecular identification. Out of 40 digestive symbiont isolates, two *Bacillus* isolates of the γ-hemolytic type, namely ALF 3 and ALF 6, showed the most potential against seven pathogenic *Vibrio* spp. isolates (OALF 1, OALF 2, OALF 3, OALF 5, WALF 7, WALF 8, and WALF 10) from the digestive tract and infected whiteleg shrimp pond water, with toxicity percentage of 86.67-100 %, α and β-hemolytic type pathogenicity in BA. Based on their anti-Vibrio activity, amylolytic and proteolytic abilities, ALF 3 and ALF 6 were identified as *Bacillus stercoris* and *Bacillus amyloliquefaciens*, respectively, with effectiveness percentages of 89.7-100 % for *B.stercoris*, 87.7-100 % for *B.amyloliquefaciens*, and 97.7-100 % for the consortium compared to the treatment without co-culture with potential bacteria against the *Vibrio* spp. population growth in TCBSA media. This study highlights the potential of whiteleg shrimp digestive symbiont bacteria to enhance immunity against vibriosis while providing safe digestive enzymatic potential for whiteleg shrimp and humans as a potential probiotic candidate.

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INTRODUCTION

Indonesia is the third-largest shrimp exporting country globally. Based on the data from the Directorate of Production and Cultivation, the most developed aquaculture type in Bengkulu Province is whiteleg shrimp (*Litopenaeus vannamei*), which is a type of shrimp that has animal protein sources and high export value in the global market, thus maintaining stable shrimp prices domestically and attractiveness as an economically stable export commodity. Whiteleg shrimp is listed as the sixth most cultivated species in the world with 5 billion tons produced in 2018, contributing 53 % of the total global aquaculture crustacean species production (Amatul-Samahah et al. 2020) and has the highest unit value of USD 26.7 billion with data from the Ministry of Marine Affairs and Fisheries of the Republic of Indonesia targeting 250 % increase in the export value and production of whiteleg shrimp by 2024 (Mustafa et al. 2023). Bengkulu Province, particularly Kaur Regency, has the potential for developing whiteleg shrimp cultivation due to its favourable water conditions. Kaur Regency, which is divided into 5 sub-districts, 3 sub-districts, and 192 villages, including Pangubaian, Linau, Cucupam, Bandar Jaya, and Air Langkap villages, is well-suited for whiteleg shrimp pond cultivation (Fernando et al. 2021).

The whiteleg shrimp is characterised by a body covered in a thin, yellowish-white chitinous exoskeleton with white legs. Whiteleg shrimp are euryhaline, thriving in a broad salinity range of 2-40 ppt, nocturnal and omnivorous. However, farming often faces production failures and shrimp mortality which are obstacles and losses due to disease attacks and antibiotic resistance in whiteleg shrimp populations. One of the challenges in whiteleg shrimp farming is the occurrence of Vibriosis caused by *Vibrio* spp. bacteria from the Vibrionaceae family. This disease can be highly detrimental to aquaculture operations, leading to mortality rates exceeding 80 % in floating net cage systems and up to 100 % during seeding or pond expansion processes. *Vibrio* spp. is a Gram-negative pathogenic bacteria, facultative anaerobic, comma-shaped structure, dimensions ranging from 1.4-2.6 µm in length and 0.5-0.8 µm in width. It does not form spores, is motile due to flagella at the cell's end, and possesses a sheath. Mortality caused by Vibriosis occurs when the shrimp are under stress conditions caused by factors such as poor pond water quality, high population density, elevated water temperature, low dissolved oxygen levels, limited water exchange, and have symptomps such as hypoxia, reddish body coloration, brownish gills, reduced appetite, lethargy, and slow swimming near the pool surface. Infections by *Vibrio* spp. in the hepatopancreas result in vacuole damage, indicating low fat and glycogen stores, trap and absorb nutrients, exhibit antibiotic resistance, and form strong associations with other bacteria or their hosts.

The use of antibiotic compounds is linked to the presence of pathogenic *Vibrio* bacteria in pond areas. Antibiotics are utilized for disease prevention, treatment, and promoting whiteleg shrimp growth. Various antibiotics commonly used can contribute to the development of antibiotic-resistant of *Vibrio*. In Brazil, approximately 70 *Vibrio* strains from water and sediment samples were found to be resistant to antibiotics such as Ampicillin, Ciprofloxacin, Chloramphenicol, Nitrofurantoin, Gentamicin, Oxytetracycline, Tetracycline, and Streptomycin. Research conducted in shrimp pond environments revealed that *V. parahaemolyticus* resistance to ampicillin and oxytetracycline at rates of 72 % and 3 % (Alloul et al. 2021).

Several developed countries have started imposing stricter quality standards on whiteleg shrimp imports from developing countries like Indonesia to enhance consumer trust in the quality and safety of fishery products. The volume of whiteleg shrimp exports from Indonesia has dropped by approximately 64 % following the introduction of a zero-tolerance policy for

antibiotic residues in mid-2001 (BPBAP Situbondo 2021). Alternative solutions are necessary in addition to environmental quality improvements, such as the utilisation of natural antibacterials as the antibiotics (Torpee et al. 2021).

The symbiotic bacteria found in the digestive system of whiteleg shrimp (*L. vannamei*) exhibit antimicrobial properties, specifically inhibiting the growth of pathogenic *Vibrio* bacteria. These bacteria belong to the *Bacillus* genus, which are rod-shaped, Gram-positive, spore-forming, catalase-positive, and oxidase-positive or negative organisms that do not hydrolyse urea. The presence of *Bacillus megaterium* in the shrimp's digestive tract can enhance microbial balance, improve feed absorption, and reduce pathogen levels by competing for nutrients and attachment sites, altering bacterial metabolism, and boosting immunity. This research aims to screen and to identify symbiotic bacteria involved in the digestion tract of whiteleg shrimp intestines, determining the potential of these symbiotic bacteria as anti-*Vibrio* agents encountered by whiteleg shrimp farmers in Kaur Regency, Bengkulu, proposing suitable solutions for these issues.

MATERIALS AND METHODS

Materials

The material used in this research was whiteleg shrimp (*L. vannamei*) in the form of the digestive tract and water from ponds in each whiteleg shrimp pond in three villages: Air Langkap, Pangubaian, and Cukoh, Kaur Regency, Bengkulu Province (Figure 1). Other materials used for screening, testing, and analysing using bacterial growth media included Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBSA), Zobell Marine Broth (ZMB), Pure Agar, and Mueller Hinton Agar (MHA).

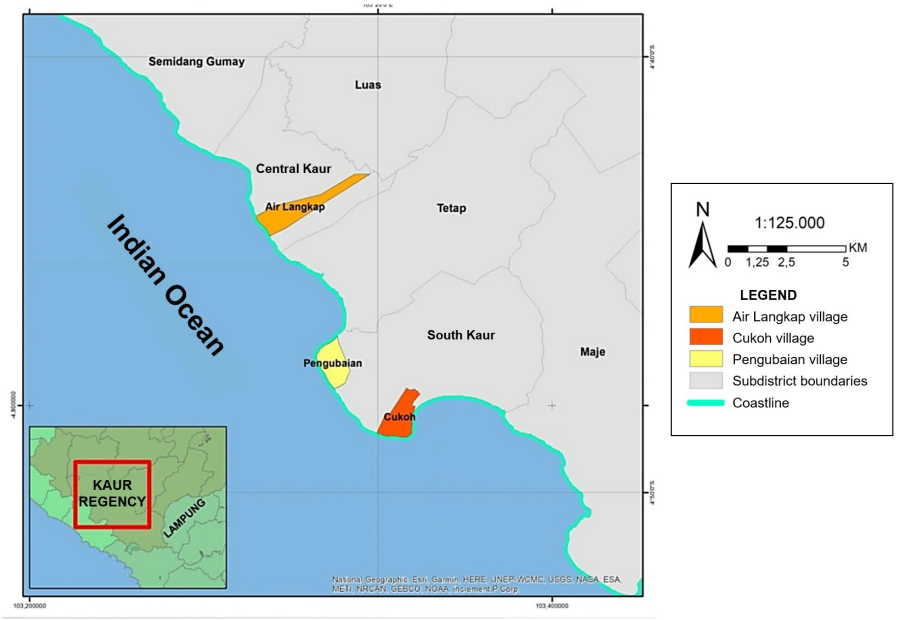

Figure 1. Location map of whiteleg shrimp sampling in Kaur Regency, Bengkulu Province.

Methods

Data collection begins with sampling of whiteleg shrimp by measuring abiotic factors (air temperature, light intensity, coordinates, salinity, water temperature, water pH, and altitude) of whiteleg shrimp ponds, isolation of symbiotic bacteria and pathogenic bacteria using serial dilution method and swab of digestive organs of whiteleg shrimp intestines infected with disease, purification, identification (morphological, Gram-staining, biochemical tests), and Anti-*Vibrio* activity tests that are symbiotic with the digestive tract of white-

leg shrimp intestines (foregut, midgut, hindgut) against pathogenic bacteria *Vibrio* spp.. Candidates that show anti-*Vibrio* activity are then further tested with toxicity, hemolysin, effectiveness, enzymatic, molecular identification with 16S rRNA gene, and data analysis.

Whiteleg Shrimp Sampling

A total of 15 whiteleg shrimp (*Litopenaeus white*) with the number of samples taken in each pond as many as 5 whiteleg shrimp with an average weight of 11.23 g were successfully collected alive from traditional ponds in Kaur Regency using purposive sampling. Fresh whiteleg shrimp samples were then taken to the Microbiology Laboratory of the Faculty of Mathematics and Natural Sciences, University of Bengkulu for further analysis. Isolation and identification of *Vibrio* spp. bacteria were carried out from the digestive tract of infected whiteleg shrimp.

Isolation and Purification

Symbiont bacteria and pathogenic *Vibrio* spp. bacteria were isolated from the digestive tract of whiteleg shrimp and water ponds by extracting the digestive tract and then cleaning the outside of the organs with sterile aquadest solution. Digestive tract was collected and cultured onto ZMA media. The bacteria were purified in new media by re-inoculating into the new ZMA media and then incubating at 28 °C for 24 hours. Purification was also conducted on Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar Media specific for pathogenic bacteria *Vibrio* spp. (Duan et al. 2020).

Morphological Identification and Gram Staining

Bacterial isolates were identified based on bacterial morphology, including appearance, shape, edges, surface, top colony colour, bottom colony colour, and bacterial colony elevation from purification result of a single isolate colonies. The staining process involved the sequential application of crystal violet reagent, iodine, 96 % alcohol, safranin, and a final rinse with water (Lay 1994). The prepared slides were then examined under a light microscope at a magnification of 1000x. Based on morphological observation and Gram-staining also biochemical test, symbiotic bacteria and pathogenic bacteria *Vibrio* spp. from the digestive tract of whiteleg shrimp were identified according to the guidelines in Bergey's Manual of Determinative Bacteriology 9th Edition and Bergey's Manual of Systematic Bacteriology 2nd Edition Volume 3rd.

Biochemical characterization

Biochemical characterisation is conducted to determine the physiological characteristics of bacteria and the closeness of bacteria at the genus level with the metabolism of a bacterium seen from the ability of a bacterium to use certain compounds as a source of carbon and energy. This includes catalase using 3 % H2O2 solution, urea using 2 % urea medium, citrate using 2 % Simmons Citrate Agar (SCA) medium, sugar fermentation (glucose, sucrose, lactose), and starch hydrolysis using Starch Agar (SA) (Lay 1994).

Anti-*Vibrio* Activity Test

A single isolate of pathogenic *Vibrio* spp. bacteria was cultured in 50 ml of MHB media and shaken for 24 hours. Then, 1 ml of the pathogenic *Vibrio* spp. culture was added to 100 mL of MHA, homogenised, and poured into a Petri dish. The symbiont bacteria isolate was inoculated into the media containing *Vibrio* spp. and incubated at 28 °C for 48 hours. Anti-*Vibrio* activity tests were carried out using isolate cultures, pellets and supernatants of symbiotic bacteria. Positive results are indicated by the formation of a clear zone around the isolate or disc paper (Hembrom et al. 2023).

Toxicity Testing Using Brine Shrimp Lethality Test (BSLT) on *Artemia salina* Larvae

The toxicity of all isolates will be detected and assessed based on the mortality rate of *Artemia salina* larvae from shrimp egg, selected by soaking them in distilled water; good eggs will sink by submerged in artificial seawater are left for 2 x 24 hours until hatch into *A. salina* larvae. Each group is divided into vials bottle consisting of 20 larvae in each 5 ml. 5 ml of artificial seawater is inoculated with the culture of the pathogenic *Vibrio* spp. bacterial isolates. Dead larvae are selected as the standard isolate to study pathogenicity or toxicity. The number of dead larvae will be counted, and the percentage of mortality obtained using the formula:

$$\text{Number of Larval Deaths (\%)} = \frac{\text{Number of Control Larvae} - \text{Number of Test Larvae}}{100}$$

The percentage of larval mortality will be used to determine the toxicity percentage using *A. salina* larvae (Mufidah & Diantari 2019).

Hemolysin of Non-Pathogenic Probiotic Bacteria and Pathogenic *Vibrio* spp.

The selected probiotic symbiont bacterial isolates and pathogenic *Vibrio* spp. were tested using blood agar test media with BA media containing 5 % goat blood. Bacteria were inoculated in the middle of the BA media. Then incubated at 37 °C for 24 hours. Hemolytic activity was indicated by the formation of clear zone around the isolate bacteria (Mohamad et al. 2020).

Effectiveness Test of Percentage Inhibition of Pathogenic Bacterial Population *Vibrio* spp. Using Liquid Culture with Potential Symbiont Bacteria

The effectiveness test of the percentage of bacterial population inhibition was conducted to determine the percentage of inhibition of the population of pathogenic bacteria *Vibrio* spp. by the symbiotic bacteria from the whiteleg shrimp digestive tract as probiotic bacteria. The test involved culturing pathogenic bacteria *Vibrio* spp. and potential symbiotic bacteria from the whiteleg shrimp digestive tract together in ZMB liquid media. The cultures were then incubated on an orbital shaker at a speed setting of 120 rpm, 30 °C for 24 hours. The experiments included control tests using liquid cultures of pathogenic bacteria *Vibrio* spp. and test treatments using liquid cultures containing pathogenic bacteria *Vibrio* spp. along with potential symbiotic bacteria. This was done to observe the percentage of population inhibition resulting from a decrease in the population of the test pathogen due to the liquid culture combination. The incubation results were followed by counting the number of colonies on agar plates to determine the Total Plate Count (TPC) population density. The calculation involved considering the number of colonies, dilution factors, and the volume of culture spread on the Petri dish to determine the percentage of inhibition of the growth of the test pathogenic bacteria *Vibrio* spp. (Amelia et al. 2020).

Enzymatic Activity Test of Probiotic Symbiont Bacteria

The enzymatic activity test of amylase activity testing was carried out using SA media. The presence of starch hydrolysis activity is indicated by the formation of a clear zone around the bacterial isolate colony after incubation for 24-72 hours at 30 °C. Protease activity testing was conducted using SMA media. Proteolytic activity is indicated by the formation of a clear zone around the colony (Joshi et al. 2022).

Molecular Identification of 16S rRNA Gene and data Analysis

Bacterial genomes were isolated using the Presto™ Mini gDNA Bacteria kit protocol (Geneaid), which involves cell lysis, DNA ligation, washing, elution, amplification, and sequencing of the 16S rRNA gene. DNA was amplified

with forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1378R (5'-GGG CGG WGT GTA CAA GGC-3') targeting a fragment of approximately ± 1300 bp. The total PCR reaction volume used was 50 µl consisting of 4 µl of genomic DNA template, 5 µl of each primer, 25 µl of GoTaq Green Mastermix 2x, and 11 µl of Nuclease-Free Water (NFW). The PCR temperature at the pre-denaturation stage was 94 °C for 5 minutes, the denaturation stage was 94 °C for 45 seconds, the annealing stage was 55 °C for 1 minute, and the elongation stage was 72 °C for 1 minute 10 seconds. The denaturation, annealing, and elongation stages were repeated 30 cycles. Then at the post-elongation PCR stage, it was 72 °C for 7 minutes, and the cooling stage was 15 °C for 15 minutes. The amplified DNA was visualized on a 1 % agarose gel stained with ethidium bromide and electrophoresed at 85 Volts for 40 minutes (Priyanto et al. 2023). Gene sequencing was carried out by PT. Genetic Science Indonesia. The 16S rRNA gene sequence was analysed using a PCR machine. The sequences were analysed using the BlastN program on the NCBI (National Centre for Biotechnology Information) website after editing with the Bioedit and MEGA 11 tools.

RESULTS AND DISCUSSION

Whiteleg Shrimp Sampling

The samples used were 15 live/fresh whiteleg shrimp (*L. vannamei*) with an average weight of 7 g to 18.78 g, as well as pond water from the vicinity of the traditional ponds. Abiotic factors of the whiteleg shrimp pond location presented in Table 1.

Isolation and Purification

Isolation of symbiont bacteria which have symbiosis with digestion of whiteleg shrimp (*L. vannamei*) and pathogenic bacteria *Vibrio* spp. was carried out by collecting samples of whiteleg shrimp using a purposive sampling method in several traditional ponds in Kaur, Bengkulu. Whiteleg shrimp that have been collected can be seen in Figure 2.

The screening process identified 40 colonies of symbiont bacteria with

Table 1. Abiotic factors for whiteleg shrimp and water samples in shrimp ponds.

No	Parameter						
	Air Temperature (°C)	Light Intensity (lux or µmol m^2s^{-1})	Coordinate	Salinity (‰)	Water Temperature (°C)	Water pH	Height (m)
1	30	6.48 x 10^4 or 1.198,8	S 04°44'53.74" E103°18'31.82"	30	31.2	6.54	33
2	30	6 x 10^4 or 1.110	S 04°46'47.83" E103°18'41.80"	34	28.5	8.11	17
3	28	6.09 x 104 or 1.126,7	S 04°48'39.94" E103°20'30.94"	31	29.9	7.76	14

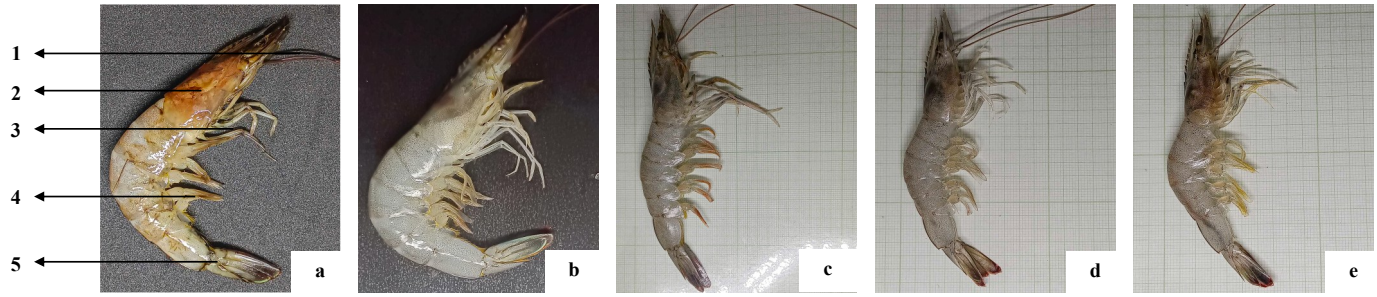

Figure 2. Whiteleg Shrimp (*L. vannamei*) samples: (a) Infected Whiteleg Shrimp with reddish body coloration showing signs of necrosis of: 1. Antenna; 2. Carapax; 3. Pereopods; 4. Pleopods; and 5. Telson, (b) Healthy/ Normal Whiteleg Shrimp, (c) Air Langkap Whiteleg Shrimp, (d) Pengubaian Whiteleg Shrimp, and (e) Cukoh Whiteleg Shrimp.

distinct morphological features, designated as ALF (Air Langkap Farm) 1-7, PNF (Pengubaian Farm) 8-30, and CKF (Cukoh Farm) 31-40. Additionally, seven *Vibrio* spp. bacterial isolates were identified as *Vibrio* spp. OALF 1, OALF 2, OALF 3, OALF 5, WALF 7, WALF 8, and WALF 10 (O: Organ and W: Water) pond sample. *Vibrio* spp. isolates were characterized by their yellow colonies on TCBSA media, a selective and differential medium designed to inhibit the growth of non-target bacteria and promote the growth of *Vibrio* spp. bacteria.

Other compositions included sodium sulphate as a source of sulphur and NaCl, which can enhance the growth of halophilic bacteria. The colour change in the TCBSA media is due to bacteria that can ferment sucrose, lowering the pH and making the media acidic. Symbiont bacteria and *Vibrio* spp. bacteria were isolated from whiteleg shrimp pond samples and pond water using the stratified dilution method and swabs. While the results of purification obtained 40 isolates of symbiont bacteria and 7 isolates of *Vibrio* spp. bacteria.

Identification and Gram-Staining

Microscopic examination of 40 symbiont bacterial isolates revealed that 26 were Gram-positive indicated by purple cells, and 14 were Gram-negative indicated by red cells. Gram-positive bacteria retain crystal violet dye appearing purple, while Gram-negative bacteria take up safranin dye, appearing red due to the dissolution of crystal violet. These color differences result from variations in bacterial cell wall structure and chemical composition. The cell wall of Gram-positive bacteria is composed of 90 % peptidoglycan. When exposed to alcohol as a bleaching solution, the cell wall dehydrates, causing the cell pores to shrink. This shrinkage reduces the absorption of the cell wall, preventing the main dye complex crystals and Lugol's solution from exiting the cell. Consequently, the safranin dye is unable to be absorbed, resulting in the bacterial cells retaining their purple colour. The cell wall of Gram-negative bacteria is composed of lipopolysaccharide and lipoprotein, which have a high lipid content. This high lipid content causes the pores of the bacterial cells to expand when exposed to alcohol, leading to the release of the crystal violet dye complex and Lugol's solution from the cell. Consequently, the bacterial cell wall absorbs the second dye, safranin (Kristiany et al. 2022).

Biochemical Characterisation

Biochemical tests support the morphological identification and Gram-staining, which indicated that the identification results of 40 isolates of symbiotic bacteria showed closeness to several genera, namely *Bacillus*, *Marinococcus*, *Sarcina*, *Vibrio*, *Commamonas*, *Micrococcus*, *Alteromonas*, *Acidovorax*, *Acidominococcus*, and *Enterobacter*. Biochemical characterisation presented in Table 2.

A total of 19 isolates had closeness to the genus *Bacillus*, 6 isolates to the genus *Marinococcus*, 4 isolates to the genus *Sarcina*, 3 isolates to the genus *Vibrio*, 2 isolates each to the genera *Micrococcus* and *Commamonas*, and 1 isolate each to the genera *Alteromonas*, *Acidovorax*, *Acidominococcus*, and *Enterobacter*. Meanwhile, the identification results of 7 isolates of *Vibrio* spp. bacteria showed that all isolates had closeness to the genus *Vibrio*, which was supported by morphological and biochemical identification characterization. The diversity of symbiotic bacteria can be seen in Figure 3.

Based on the identification, the *Bacillus* genus is the dominant genus found in the symbiotic bacteria of the digestive tract of whiteleg shrimp, which are then referred to as *Bacillus* sp. 1 – *Bacillus* sp. 19 with rod- shaped, uses citrate as a carbon source, ferments glucose and sucrose, hydrolyses starch, is positive for catalase, motile, and commonly found in the digestive

Table 2. Biochemical Characterisation of Symbiotic Bacteria.

No	Isolate code	Species name	Biochemical test								
			Gram	C	U	Ci	G	L	S	M	SH
1	ALF 1	*Vibrio* sp. 1	-	+	-	-	+	-	+	+	-
2	ALF 2	*Acidovorax* sp. 1	-	+	+	-	-	-	-	+	-
3	ALF 3	*Bacillus* sp. 1	+	-	-	+	+	-	+	+	+
4	ALF 4	*Micrococcus* sp. 1	+	+	-	+	+	-	-	+	-
5	ALF 5	*Acidaminococcus* sp. 1	-	-	-	-	-	-	-	+	-
6	ALF 6	*Bacillus* sp. 2	+	-	-	-	-	-	-	+	+
7	ALF 7	*Marinococcus* sp. 1	+	+	-	+	+	-	+	+	-
8	PNF 8	*Vibrio* sp. 2	-	-	-	+	+	-	+	+	-
9	PNF 9	*Bacillus* sp. 3	+	+	-	-	+	-	-	+	-
10	PNF 10	*Sarcina* sp. 1	+	-	-	-	-	-	-	+	-
11	PNF 11	*Enterobacter* sp. 1	-	+	-	-	+	-	+	+	+
12	PNF 12	*Micrococcus* sp. 2	+	+	-	-	-	-	+	+	+
13	PNF 13	*Commamonas* sp. 1	-	-	-	-	+	-	+	+	-
14	PNF 14	*Bacillus* sp. 4	+	-	-	-	+	-	+	+	+
15	PNF 15	*Bacillus* sp. 5	-	-	-	-	+	-	+	+	-
16	PNF 16	*Bacillus* sp. 6	+	+	-	-	+	+	-	+	+
17	PNF 17	*Bacillus* sp 7	+	-	-	-	+	+	-	+	-
18	PNF 18	*Marinococcus* sp. 2	+	-	-	-	+	+	+	+	-
19	PNF 19	*Sarcina* sp. 2	+	+	-	-	+	-	+	+	-
20	PNF 20	*Marinococcus* sp. 3	+	-	-	-	+	+	+	+	-
21	PNF 21	*Bacillus* sp. 8	+	-	-	-	+	-	+	+	-
22	PNF 22	*Bacillus* sp. 9	-	-	-	-	+	+	-	+	+
23	PNF 23	*Vibrio* sp. 3	-	-	-	-	+	-	+	+	-
24	PNF 24	*Marinococcus* sp. 4	+	-	-	-	+	+	+	+	+
25	PNF 25	*Bacillus* sp. 10	+	-	-	+	+	-	+	+	-
26	PNF 26	*Bacillus* sp. 11	-	+	-	-	+	+	+	+	+
27	PNF 27	*Sarcina* sp. 3	+	+	-	+	+	-	+	+	-
28	PNF 28	*Bacillus* sp. 12	-	-	-	-	+	+	+	+	-
29	PNF 29	*Bacillus* sp. 13	+	-	-	-	+	-	+	+	-
30	PNF 30	*Bacillus* sp. 14	-	+	-	+	+	-	+	+	-
31	CKF 31	*Bacillus* sp. 15	+	+	-	+	-	-	-	+	-
32	CKF 32	*Sarcina* sp. 4	+	-	-	+	-	-	-	+	+
33	CKF 33	*Bacillus* sp. 16	+	+	-	-	+	-	-	+	-
34	CKF 34	*Bacillus* sp. 17	+	+	-	-	-	-	-	+	-
35	CKF 35	*Marinococcus* sp. 5	+	+	-	-	-	-	-	+	-
36	CKF 36	*Marinococcus* sp. 6	+	+	-	+	-	-	-	+	+
37	CKF 37	*Alteromonas* sp. 1	-	+	-	-	+	-	+	+	-
38	CKF 38	*Bacillus* sp. 18	+	+	-	-	-	-	+	+	-
39	CKF 39	*Commamonas* sp. 2	-	+	-	-	-	-	-	+	-
40	CKF 40	*Bacillus* sp. 19	+	+	-	-	-	-	-	+	-

Note. ALF= Air Langkap *Farm*, PNF= Pengubaian *Farm*, CKF= Cukoh *Farm*, Gram= Gram-staining, C= Catalase, U=Urea, Ci= Citrate, G= Glucose, L= Lactose, S= Sucrose, M= Motility, SH= Starch Hydrolysis, + = Positive, - = Negative.

tract, indicating that feed supplemented with probiotics can enhance the competitive microbial interactions in the gut and protect and enhance the microbiota that can be used as feed probiotics to produce essential micronutrients in feed and aid in digestion by breaking down nutrients such as carbohydrates, proteins, and lipids by producing extracellular enzymes (Chen et al. 2021).

Anti-*Vibrio* Activity Test

The activity of anti-*Vibrio* compounds produced by bacterial isolates symbiotically associated with the digestive tract of whiteleg shrimp is characterized

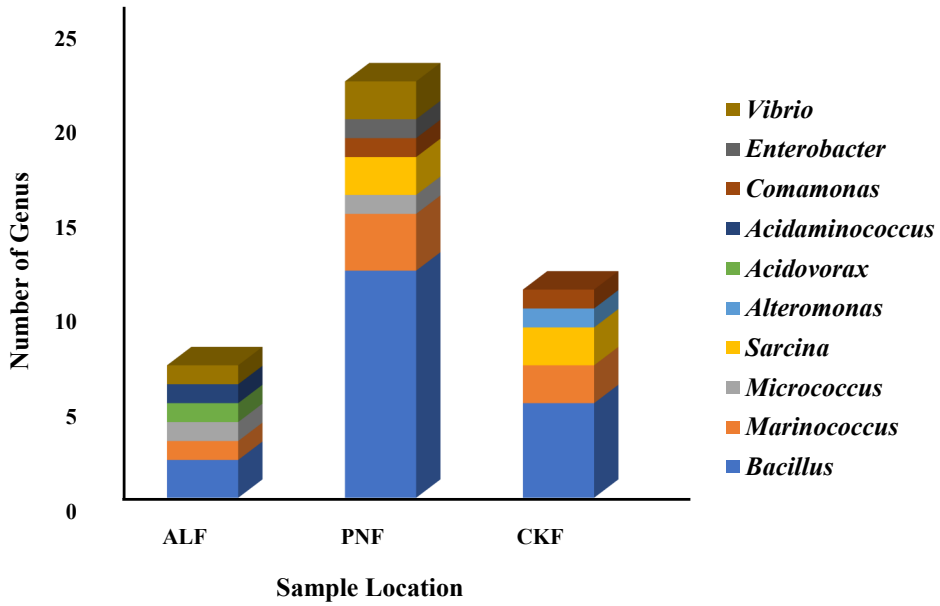

Figure 3. Distribution of diversity of symbiont bacteria from the digestive tract of whiteleg shrimp (*L. vannamei*) in Kaur Regency: ALF (Air Langkap Farm), PNF (Pengubaian Farm), and CKF (Cukoh Farm).

by the formation of clear zone around the isolate cultures or paper discs, indicating that the compounds produced can inhibit the growth of the pathogenic bacteria *Vibrio* spp. using isolate cultures, pellets and supernatants shown in Table 3 and Figure 4.

Table 3. Anti-*Vibrio* activity test using culture, pellets and supernatants of symbiotic bacteria.

No	Isolate code	Vibrio pathogen	Anti-*Vibrio* test (mm)		
			Culture	Pellets	Supernatants
1	ALF 3	OALF 1	+	5.85 ± 0.10	3.35 ± 0.10
		OALF 2	-	0.00 ± 0.00	0.00 ± 0.00
		OALF 3	+	5.38 ± 0.03	4.05 ± 0.25
		OALF 5	+	8.35 ± 1.00	0.00 ± 0.00
		WALF 7	+	0.00 ± 0.00	2.13 ± 0.28
		WALF 8	-	0.00 ± 0.00	0.00 ± 0.00
		WALF 10	+	7.80 ± 0.05	7.80 ± 0.05
2	ALF 6	OALF 1	+	11.05 ± 0.15	10.30 ± 0.75
		OALF 2	-	0.00 ± 0.00	0.00 ± 0.00
		OALF 3	-	0.00 ± 0.00	0.00 ± 0.00
		OALF 5	-	0.00 ± 0.00	0.00 ± 0.00
		WALF 7	+	3.23 ± 0.03	2.13 ± 0.18
		WALF 8	-	0.00 ± 0.00	0.00 ± 0.00
		WALF 10	-	0.00 ± 0.00	0.00 ± 0.00

Note. Anti-*Vibrio* activity test using culture: (+)= positive with forming a clear zone; (-)= Negative without forming a clear zone.

Isolation of symbiotic bacteria was tested for anti-*Vibrio* activity against 7 *Vibrio* spp. bacteria, namely *Vibrio* spp. OALF 1, OALF 2, OALF 3, OALF 5, WALF 7, WALF 8, and WALF 10. Out of the 40 symbiotic isolates tested, 17 isolates were found to have the potential to inhibit the growth of *Vibrio* spp. bacteria, namely isolates ALF 1-7, PNF 8, 11, 17, 20, 23, 24, 25, 27, 30, and CKF 32, including 2 target bacteria *Bacillus* spp., namely ALF 3 and ALF 6. Isolates that did not inhibit are suspected because *Vibrio* spp. have a high level of pathogenicity and possess pathogenic genes such as the TDH gene that can stimulate the production of toxins such as Thermostable Direct He-

molysin (TDH) and the TRH gene that produces the toxin TDH-Related Hemolysin (TRH) (Leber 2016).

The testing results of pellets from 17 isolates of symbiotic bacteria in the digestive tract of whiteleg shrimp in the bacterial isolate culture test produced 16 potential symbiotic bacterial isolates in the pellet activity test. The differences in growth that occur between one bacterium and another are caused by the different abilities of bacteria to reproduce, depending on the growing media and available nutrients. Bacterial growth factors also depend on pH and temperature (Dayalan et al. 2022) also influenced by the enzyme content, which affects the production anti-*Vibrio* compounds.

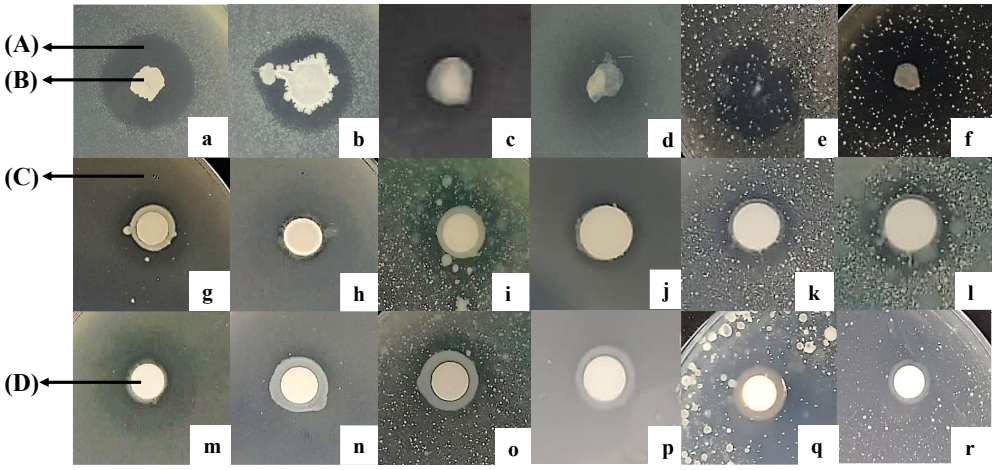

Figure 4. Anti-*Vibrio* activity test using culture: (a-c) ALF 3, ALF 6, PNF 11 against *Vibrio* spp. OALF 1, (d) ALF 3 against *Vibrio* spp. OALF 3, (e-f) ALF 6 and PNF 11 against *Vibrio* spp. WALF 7; using pellets: (g-h) PNF 11 and PNF 23 against *Vibrio* spp. OALF 1, (i) PNF 11 against *Vibrio* spp. OALF 3, (j) ALF 3 against *Vibrio* spp. WALF 5, (k) PNF 23 against *Vibrio* spp. WALF 7, (l) PNF 23 against *Vibrio* spp. WALF 10; using supernatants: (m-n) ALF 6 and PNF 11 against *Vibrio* spp. OALF 1, (o) PNF 11 against *Vibrio* spp. OALF 3, (p) PNF 11 against *Vibrio* spp. OALF 5, (q) ALF 2 against WALF 8, (r) PNF 24 against *Vibrio* spp. WALF 10 which is indicated by (A) clear zone (B) bacterial cell culture (C) media and (D) disc paper.

Antibacterial compounds produced by bacteria can be excreted by cells into the medium (extracellularly) as a whole (or in part) and can also be found within the cell (intracellularly). According to Proespraiwong et al. (2023), anti-*Vibrio* compounds can be excreted by bacterial cells into the media extracellularly either entirely or partially and can also be found within bacterial cells intracellularly. Anti-*Vibrio* compounds obtained from the pellet are compounds bound to cell residues. Meanwhile, the supernatant contains polar compounds that cause the media during liquid culture to be extracted from the bacterial cells, so the anti-*Vibrio* compounds obtained from the supernatant are not bound to bacterial cells.

Supernatant testing of 17 isolates of symbiotic bacteria from the digestive tract of whiteleg shrimp in pond culture resulted in 16 potential symbiotic bacterial isolates in activity tests with the inhibitory mechanisms include inhibiting bacterial cell wall synthesis, bacterial protein synthesis, folate synthesis, DNA synthesis, and altering cell membrane permeability.

Toxicity Test Using BSLT on *A. salina* Larvae

The toxicity test of pathogenic *Vibrio* spp. bacteria showed that isolates *Vibrio* spp. with codes *Vibrio* spp. OALF 2 and *Vibrio* spp. WALF 7 were *Vibrio* spp. bacteria with the highest average larval death/kill percentage of 100 % in each replication. Meanwhile, the lowest average larval death percentage was

shown in the isolate *Vibrio* spp. with code *Vibrio* spp. OALF 1 at 86.67 %. This test was supported by the inhibition activity test of symbiont bacteria against *Vibrio* spp. bacteria using symbiont bacteria culture test, supernatant, and pellet, which showed that isolates *Vibrio* spp. OALF 2 and *Vibrio* spp. WALF 8 were *Vibrio* spp. bacteria that were more resistant and less inhibited by symbiont bacteria, while *Vibrio* spp. OALF 1 was the *Vibrio* spp. bacteria most inhibited by symbiont bacteria. This indicates that the seven isolates of pathogenic *Vibrio* spp. bacteria have a toxicity level range of 86.67 – 100 % on *A. salina* larvae within 24 hours can be seen in Figure 5.

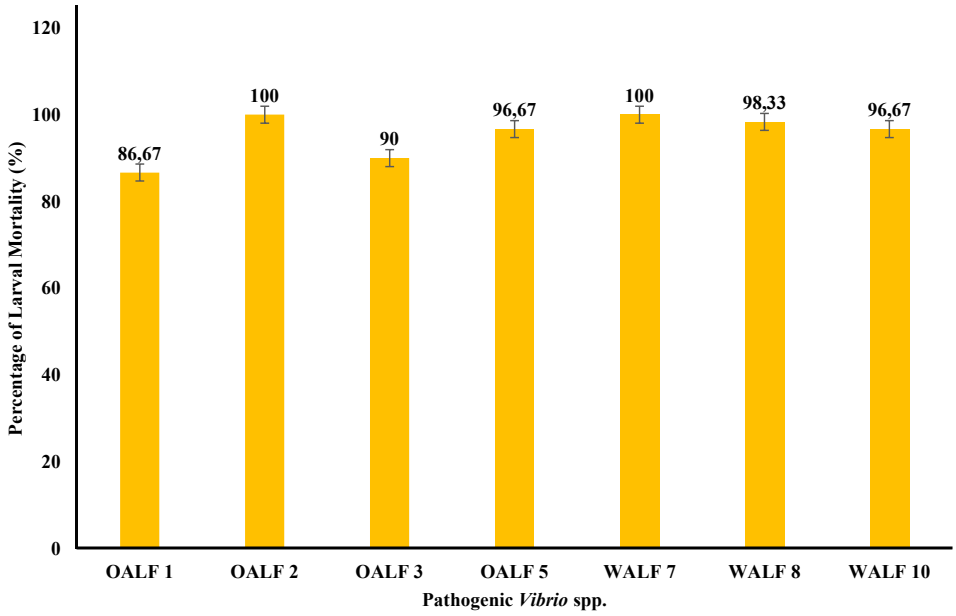

Figure 5. Percentage of Mortality of *A. salina* Larvae in Treatment of Pathogenic Bacteria *Vibrio* spp. OALF 1 – WALF 10.

BLST is one form of the interaction between pathogens and their hosts. The interaction of the pathogenic *Vibrio* spp. bacteria with crustaceans, generally involve entering and colonizing the whiteleg shrimp organism through the digestive tract or through wounds on the outer shell. Pathogenic *Vibrio* spp. bacteria attach to various tissues, including the hepatopancreas, gills, and hemolymph. The ability of these bacteria to adhere to tissues is facilitated by specific adhesive factors that enhance colonization efficiency, along with virulence factors that contribute to their pathogenicity (Devi et al. 2019). Virulence factors possessed by pathogens generally include the ability to produce toxins, form Quorum Sensing (QS) and form biofilms. Pathogenic bacterial species *Vibrio* spp. produce toxins that can damage host tissues, such as *V. parahaemolyticus* known to produce hemolysin and protease that can cause tissue necrosis and disrupt normal physiological functions in shrimp.

Hemolysin of Non-Pathogenic Probiotic Bacteria and Pathogenic *Vibrio* spp.

The hemolysis test of symbiotic bacteria producing anti-*Vibrio* compounds, which are candidate non-pathogenic symbiotic bacteria, there were a total of 2 isolates: ALF 3 and ALF 6 belonging to the genus *Bacillus*, specifically *Bacillus* sp. 1 and *Bacillus* sp. 2. Both isolates have the hemolytic type γ (gamma) hemolysis. Gamma hemolysis is characterized by bacteria not producing hemolysin enzymes, resulting in no lysis, no clear zone formation, and no color change around the bacterial isolates on the media.

Based on this research, two potential symbiotic bacterial isolates ALF 3 and ALF 6 showed γ-hemolytic that do not form a clear zone around the iso-

lates, thus serving as safety indicators and effectively as probiotic candidates that play a crucial role in modulating the shrimp gut microbiota, contributing to better shrimp growth by improving digestion and nutrient absorption safely without posing additional risks.

The hemolysis test of seven pathogenic bacteria *Vibrio* spp. exhibited α-hemolytic (OALF 1, OALF 3, OALF 5, and WALF 10) display a green-black colour around the isolate due to the H_2O_2 product from pathogenic *Vibrio* spp. bacteria converting Hemoglobin (Hb) in red blood cells to methemoglobin (MetHb) (partial lysis of red blood cells), while the β-hemolytic (OALF 2, WALF 7, and WALF 8) forming clear zone as result of red blood cell lysis by hemolysin, destroys cells until hemoglobin is completely denatured without producing a colored product (Amiin et al. 2023). This indicates relationship between hemolytic and toxicity, OALF 2 and WALF 7 exhibiting 100 % toxicity on *A. salina* due to their β-hemolytic ability. The hemolysis test can be seen in Figure 6.

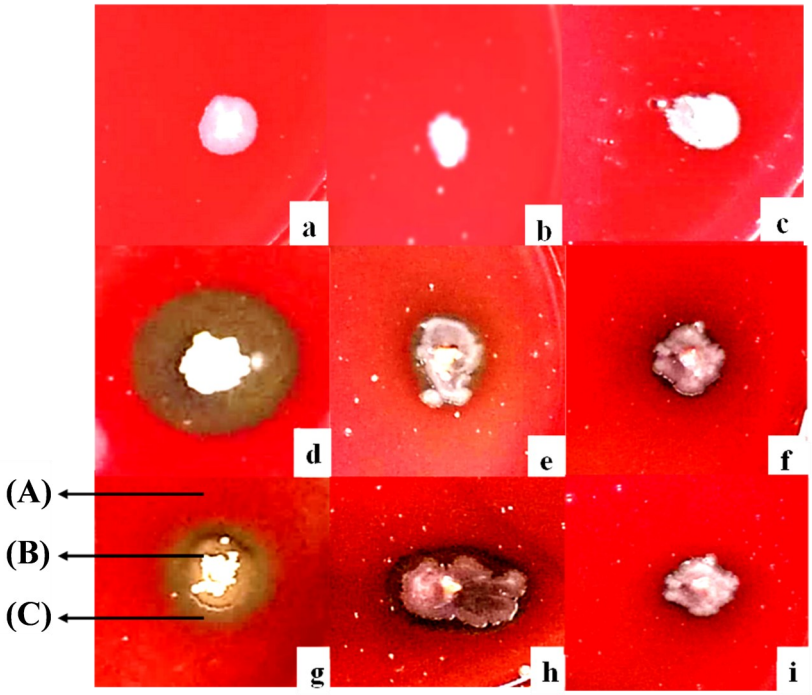

Figure 6. Hemolysin test of non-pathogenic probiotic bacteria producing anti-*Vibrio* compounds on isolates: a) ALF 3 and b) ALF 6; Pathogenic bacteria *Vibrio* spp. on isolates: c) OALF 1; d) OALF 2; e) OALF 3; f) OALF 5; g) WALF 7; h) WALF 8; and i) WALF 10.

Effectiveness Test of Percentage Inhibition of Pathogenic Bacterial Population *Vibrio* spp. Using Liquid Culture with Potential Symbiont Bacteria

In the effectiveness test, the percentage inhibition of the population of potential probiotic symbiont bacteria against the pathogenic bacteria *Vibrio* spp. in the control and treatment tests after the addition of potential symbiont bacteria *Bacillus* spp. The treatment of liquid culture with *Bacillus* sp. 1 shows the percentage of inhibition of the growth of pathogenic bacteria *Vibrio* spp. ranging from 89.7 to 100 % (Figure 7a), the liquid culture with *Bacillus* sp. 2 shows the percentage of inhibition of the growth of pathogenic bacteria *Vibrio* spp. ranging from 87.7 to 100 % (Figure 7b), and the liquid culture with *Bacillus* sp. 1 and *Bacillus* sp. 2 shows the percentage of inhibition of the growth of pathogenic bacteria *Vibrio* spp. ranging from 97.7 to 100 % (Figure 7c). The percentage of inhibition of pathogenic bacteria *Vibrio* spp. in culture with potential *Bacillus* spp. bacteria overall can be seen in Figure 7.

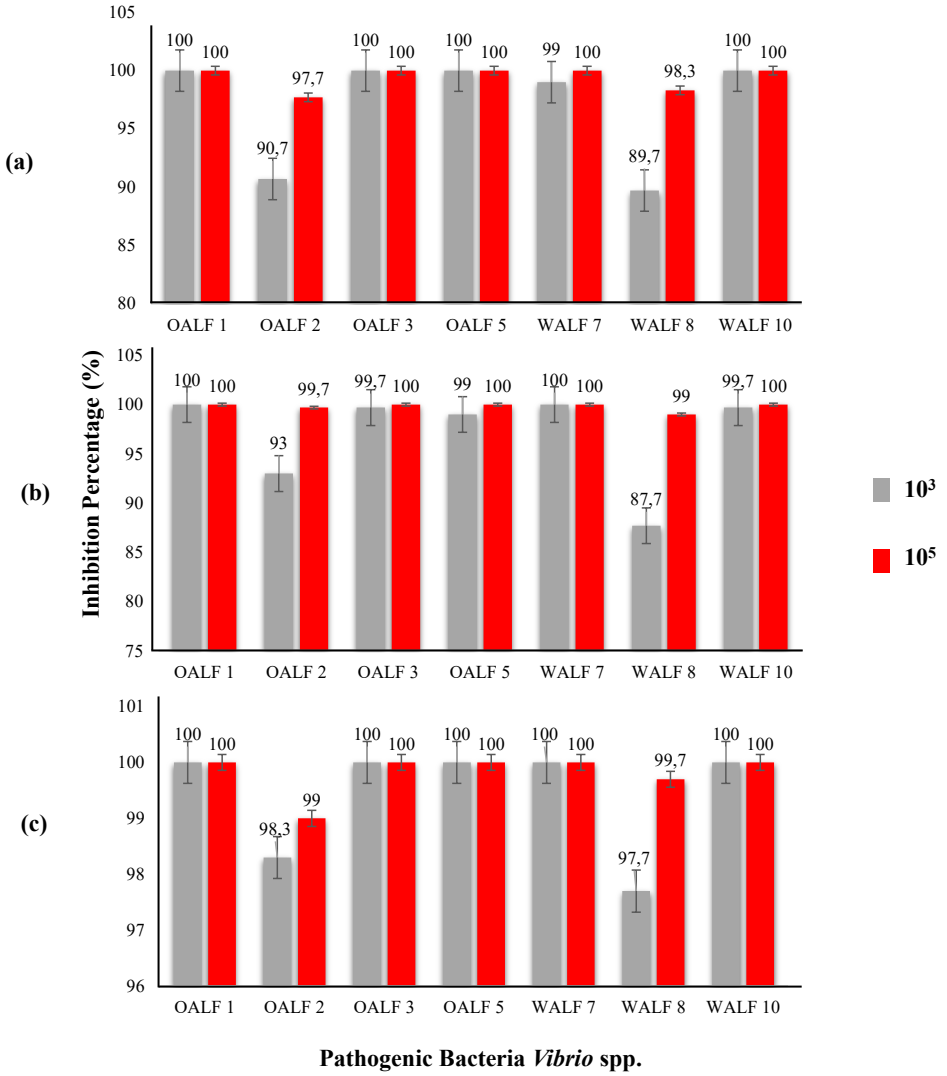

Figure 7. Percentage graph of inhibition of pathogenic bacteria *Vibrio* spp. culture with potential *Bacillus* spp. bacteria: (a) *Bacillus* sp. 1, (b) *Bacillus* sp. 2, and (c) consortium of *Bacillus* sp. 1 and *Bacillus* sp. 2.

Effective inhibition is inhibition with effective probiotic adhesion properties that show strong adhesion to intestinal mucus can enhance their ability to multiply in the host and provide inhibitory effects against pathogens. According to Rusmana et al. (2021a), *Bacillus* spp. isolates from whiteleg shrimp ponds have high anti-quorum sensing activity on *V. parahaemolyticus* for inhibition activity of AQS up to 75 %, such as *B. cereus*, *B. thuringiensis*, and B. *velezensis* bacteria that have confirmed the aiiA gene encoding the AHL-lactonase enzyme as an AQS potentially for application in controlling AHNPD disease in shrimp. Besides, inhibition activity of selected *Bacillus* sp. Lts40 against *V. harveyi* was found with a high inhibition (88.7 %) confirmed have a protein fraction with a molecular weight of 47.38 kDa classified as a class III bacteriocin protein (> 30 kDa) as an anti-*Vibrio* (Rusmana et al. 2021b).

The highest effectiveness was obtained in the culture with a consortium of *Bacillus* sp. 1 and sp. 2, which showed the best results, combination of various *Bacillus* spp. strains showed a synergistic effect that effectively inhibited the growth of *Vibrio* spp. pathogenic bacteria beyond what was achieved by individual strains, this action arises from the production of various compounds by different species that can target various types within the pathogenic *Vibrio* spp. bacterial cells opens up opportunities for the development of multi-strain probiotic formulations candidates.

Enzymatic Activity Test of Probiotic Symbiont Bacteria

Enzymatic activity of symbiotic bacteria in whiteleg shrimp plays a crucial role in enhancing digestive function and nutrient absorption, contributing to the health and growth of whiteleg shrimp. Microbiota in the digestive tract play a vital role in breaking down complex compounds (macromolecules) in feed into simpler compounds (micromolecules) for easier digestion. Based on the amylase and protease test shown in Figure 8.

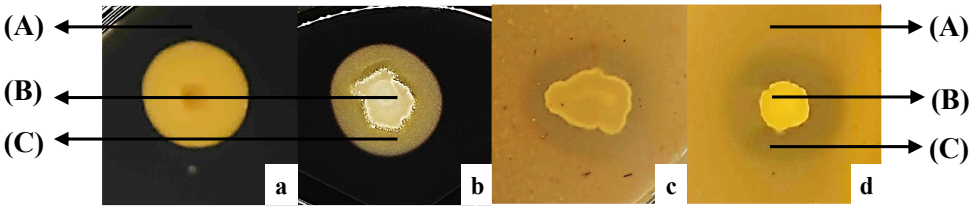

Figure 8. Enzymatic activity of non-pathogenic *Bacillus* spp. in amylase test: (a-b) ALF 3 and ALF 6; protease test: (c-d) ALF 3 and ALF 6 which is indicated by (A) SA/SMA media (B) bacterial cell culture and (C) clear zone

Figure 8 shows that the symbiotic bacteria *Bacillus* sp. 1 and *Bacillus* sp. 2 have amylase activity, indicated by the formation of a clear zone like *B. amyloliquefaciens* bacterial strain is capable of producing amylase and improving carbohydrate digestion by increasing the efficiency of glucose availability and obtaining a better feed ratio for whiteleg shrimp, enabling the conversion of feed into body mass more efficiently. Enzymatic activity facilitates digestion in enhancing nutrient absorption through the intestinal wall, shrimp growth performance, and disrupting the formation of pathogenic biofilms, making it difficult for pathogens to colonise the intestines, which can affect gut microbiota by promoting beneficial bacteria that thrive on simple sugars released during starch digestion. These changes can help suppress pathogenic bacteria such as *Vibrio* spp. and contribute to a healthier gut environment (EL-Sayed et al. 2022).

Bacillus sp. 1 and *Bacillus* sp. 2 bacteria have proteolytic activity to break down proteins into peptides and amino acids to facilitate the utilisation of protein. Shrimp supplementation with protease enzymes has been proven to enhance disease resistance by facilitating melanin synthesis and pathogen encapsulation, when infected by *V. parahaemolyticus* show lower mortality rates and higher shrimp defence that maintain intestinal morphology by increasing the surface area of microvilli and nutrient absorption capacity (Afrin et al. 2024).

Molecular Identification of 16S rRNA Gene and data Analysis

Based on 3 anti-*Vibrio* activity tests using culture isolates, pellets, and supernatants, two symbiotic bacterial isolates were obtained that have the potential to inhibit pathogenic *Vibrio* spp. bacteria in the digestive tract of whiteleg shrimp ponds ALF 3 and ALF 6. The results of PCR 16s rRNA after electrophoresis showed a DNA band length of 1300 as seen in Figure 9, and the identification based on BLASTn can be seen in Table 4.

Table 4 show the identification of bacterial isolates ALF 3 showed species homology based on the BLASTn NCBI site with *Bacillus stercoris* species, specifically strains 24177, V1194, and 23151, with a Query Cover value of 100 % and a similarity above 75 %, specifically 99.92 %. Bacterial isolate ALF 6 exhibited species homology with *Bacillus amyloliquefaciens* species, specifically strains RESI-50, 3820, and SQ-2, with a Query Cover value and similarity reaching 100 %. The constructing a phylogenetic tree of potential symbiotic bacteria are shown in Figure 10, while the variables and conservative sites of the 16S rRNA gene can be found in Table 5.

Table 4. The identification results of symbiotic bacterial isolates in the selected shrimp gut based on BLASTn from the NCBI site.

Isolate	Homology	Query Cover (%)	E-Value	Similarity (%)	Access Number
ALF 3	Bacillus stercoris strain 24177	100	0.0	99.92	OR437177.1
	Bacillus stercoris strain V1194	100	0.0	99.92	OR432101.1
	Bacillus stercoris strain 23151	100	0.0	99.92	OR432081.1
ALF 6	Bacillus amyloliquefaciens strain RESI-50	100	0.0	100	MT542326.1
	Bacillus amyloliquefaciens strain 3820	100	0.0	100	MT538668.1
	Bacillus amyloliquefaciens strain SQ-2	100	0.0	100	MN922942.1

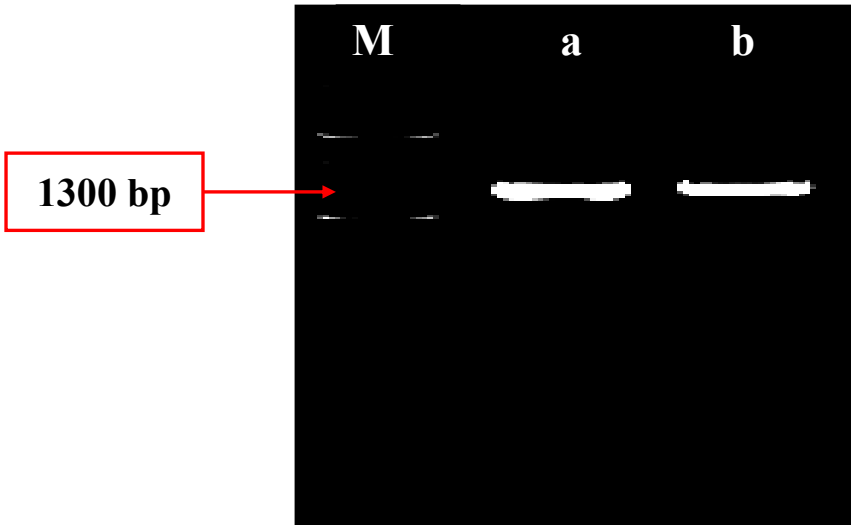

Figure 9. The electrophoresis visualisation results of the 16S rRNA gene showed a DNA band length of 1300 bp (M) Marker Ladder 1Kb in the symbiotic bacterial isolates (a) ALF 3 and (b) ALF 6.

Table 5. Variation and conservative sites of the 16S rRNA gene of ALF 3 and ALF 6 isolates.

Spesies	Conserved (C)	Variation Site			Nucleotide Composition (%)			
		Vi	Pi	S	A	T	G	C
ALF 3	952	376	290	86	24.9	20.1	31.8	23.2
ALF 6					25.3	19.9	31.7	23.2

Note: C = Conservative Site, Vi = Variable Site, Pi = Information Parsimony Site, S = Singleton, A = Adenine, T = Thymine, G = Guanine and C = Cytosine.

Based on Table 5, the data shows the variation in sites with the nucleotide composition of bacterial isolates of the ALF 3 and ALF 6 species According to the research report, to maximize the base pairing of the 16S rRNA gene, the amount of guanine must be higher than cytosine, while the amount of adenine must be higher than timine. Nitrogen base pairing bond influences the nucleotide composition to enhance and to stabilise the 16S rRNA gene. If this balance is disrupted, it often leads to inconsistent or contaminated results.

The construction of the phylogenetic tree reveals ALF 3 refers related to *Bacillus stercoris*, while ALF 6 similarities to *Bacillus amyloliqufaciens* with a bootstrap value of 100 % at C1, specifically within the Firmicutes Phylum. ALF 3, identified as a *B. stercoris* species (Figure 10), exhibits anti-*Vibrio* activity, indicating a broad spectrum of antibacterial activity against major aquaculture pathogens of the *Vibrio* genus. Notably, *Bacillus stercoris* MBTDCMFRI Ba37 strain has been utilised as a biocontrol agent in fisheries cultivation, which is responsible for its primary antibacterial activity. ALF 6 *Bacillus amyloliquefaciens* acts as anti-*Vibrio* agent utilising extracellular enzyme capabilities as probiotics for the prevention of aquatic cultivation diseases. The potential symbiotic bacteria isolate from the digestive tract of whiteleg shrimp expected to be a source of new bioactive anti-*Vibrio* compounds for further development as an alternative probiotic. This can be achieved by creating a consortium of potential symbiotic bacteria isolates from the digestive tract as a probiotic feed for whiteleg shrimp in the future as a preventive measure for traditional ponds in Kaur, Bengkulu, Indonesia.

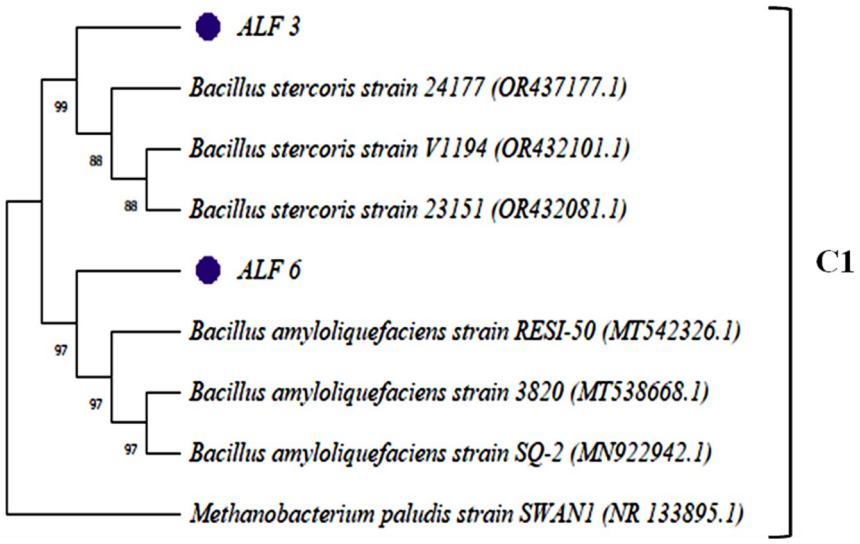

Figure 10. Phylogenetic tree construction using the Neighbor-Joining Tree method with 1000x replication.

The ability to harness this synergistic effect paves the way for the development of multi-strain probiotic formulations aimed at controlling pathogenic *Vibrio* spp. bacteria in aquaculture can provide stronger defense against infections while improving the health and growth of shrimp (Taheri-Araghi 2024). Probiotics can be administered orally with feed (including bioencapsulation with live food vectors such as Artemia, directly into water as purified cultures) or in fermented growth media, for example, soybean meal fermented with *Bacillus subtilis* E20. Similarly, probiotics can be given in combination with complementary prebiotics or indigestible food substances that provide beneficial effects to the host by selectively stimulating the growth and/or activity of one or more bacteria in the large intestine to form a treatment known as symbiotic interaction.

The digestive symbiotic bacteria of whiteleg shrimp from traditional ponds in Kaur, Bengkulu, Indonesia have been identified candidate symbiont bacteria with the potential to be used as probiotics due to their anti-*Vibrio* and enzymatic activities. Further research analyse the profile of anti-*Vibrio* compounds using GC-MS and/or LC-MS methods. Additionally, in vivo testing of potential symbiont bacterial consortia will be conducted on whiteleg shrimp specimens as a practical applications of these symbiotic bacteria as probiotics in aquaculture with formulations orally with feed or directly in water.

CONCLUSIONS

Isolation of symbiotic bacteria and pathogenic *Vibrio* spp. bacteria from the digestive tract of whiteleg shrimp yielded 40 isolates of symbiotic bacteria and 7 isolates of pathogenic *Vibrio* spp. bacteria, resulting in a total of 10 genera (*Bacillus*, *Marinococcus*, *Sarcina*, *Vibrio*, *Commamonas*, *Micrococcus*, *Alteromonas*, *Acidovorax*, *Acidominococcus*, and *Enterobacter*), with *Bacillus* being the most abundant genus and pathogenic bacteria belonging to the *Vibrio* genus. The research identified two potential isolates: ALF 3 and ALF 6, which exhibited characteristics of *Bacillus* sp. 1 and *Bacillus* sp. 2 with γ-hemolytic activity, showing promise as probiotics producing anti-*Vibrio* compounds against *Vibrio* spp. with α-hemolytic pathogenicity (*Vibrio* spp. OALF 1, OALF 3, OALF 5, and WALF 10) and β-hemolytic (*Vibrio* spp. OALF 2, WALF 7, and WALF 8). The highest inhibition effectiveness was obtained with the consortium treatment of *Bacillus* sp. 1 and *Bacillus* sp. 2, which could reduce and eliminate the growth of pathogenic *Vibrio* spp. bacteria. Their enzymatic abilities in amylase and protease can enhance digestive functions for nutrient absorption through the intestinal wall and improve resistance to disease infections. Molecular identification based on the 16S rRNA encoding gene revealed that ALF 3 belonged to *Bacillus stercoris* and *Bacillus amyloliquefaciens* with anti-*Vibrio* and enzymatic activities as potential aquaculture probiotic bacteria candidates.

AUTHOR CONTRIBUTION

R.H.W, R.W, S.S, I.R, and R.L conducted the conception of this study. R.H.W, R.W, I.R, S.S, and R.L designed the study. R.W conducted the data acquisition. All authors conducted the data analysis and interpretation. All authors wrote the original draft of the MS. R.H.W, R.W and P.P.R. revised and proofread the MS. P.P.R. Assisted in the analysis and writing of the BLST Artemia Larvae test data and edited the manuscript during the revision stage. A.R. assisted in the analysis of the direct liquid culture challenge test between the pathogen and potential symbiont bacteria and interpreted the results in the form of figures or graphs.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest during the research.

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