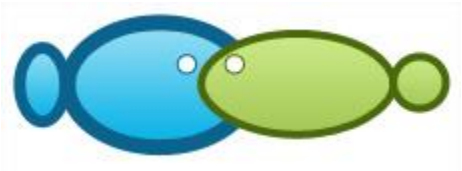

The potential of endogenous bacteria as probiotics to enhance survival, growth, and immunity of cultured spiny lobster *Panulirus homarus* (Linnaeus 1758)

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Abstract. This study aimed to identify potential strains of endogenous bacteria as probiotics and apply them in the diet of spiny lobsters to enhance survival, growth, and health status under culture conditions. Wild *Panulirus homarus* (Linnaeus 1758) lobsters were collected from the coastal waters of the Jembrana District of Bali, Indonesia. From them, intestinal bacteria were isolated, identified, and characterized, followed by enzymatic hydrolysis tests to select candidates of bacteria that could be used as probiotics. Spiny lobsters weighing 70.34±12.03g were cultured in concrete tanks of 4 m^3 capacity at a density of 15 individuals m^{-3}. Six tanks were used to feed the lobsters with a moist pellet diet supplemented with probiotics (A) or the same diet without probiotics (B), and each treatment had two replications. The research identified four bacterial strains as potential probiotics: *Photobacteria damselae* N-5, *Bacillus subtilis* C-1**,** *Bacillus oceanisediminis* H-3, and *Bacillus amyloliquefaciens* I-5. These four bacteria were combined and applied to lobsters as a dietary supplement. Growth of lobsters fed with supplemented probiotics (A) was higher (198.21g) than the control (B) (169.76 g), while survival was similar. The immune response of lobster-fed diet A was 18 times that of B after challenging with MHD, especially for target gene ALF-2, while for ProPO, CP, and GPO, the increase was 13, 35, and 94 times. Applying this probiotic to the diet could increase lobster culture's growth and immunity.
Key Words: Spiny lobster, probiotic, survival, growth, immunity.

Introduction. Lobsters are a high-value crustacean used as an export commodity for Indonesia. Most of the lobster production for export is wild-caught, but the fishery has been subject to poor management and destructive practices, and fishery volume has decreased in recent years. Global production of spiny lobster in 2014 was 306 thousand tons valued at more than US $2.7 billion (FAO 2016). Meanwhile, marketable lobster production from aquaculture is around 1500 tons per year. (Priyambodo et al 2020). Lobster aquaculture within Indonesian waters has remained quite small, with minor production in certain areas such as Lombok, South Sulawesi, South-East Sulawesi, North Sumatra, and Aceh using marine net cages (Priyambodo 2015). In 2022, Indonesia exported around 25.7 million USD worth of lobster, which decreased from the previous year. The production of lobster larvae in Indonesia is concentrated around the northern coasts of Java and Lombok islands. Lobster production is up 26% year-over-year through November 2023. The global lobster market was valued at US$7.6 billion in 2023. However, with the increasing demand for lobster, many producers have turned to aquaculture to meet this demand. Aquaculture is expected to produce 58% of the fish used for human consumption by 2029, up from 53% in 2017–2019. Aquaculture allows for the controlled cultivation of lobsters, which can be raised in large quantities and harvested at specific times to meet market demand. This has led to a more consistent

supply of lobster throughout the year than during the traditional lobster season (Polaris Market Research 2023).

In contrast, Vietnam has developed lobster culture since 1995 and is now the world leader in lobster aquaculture production. Although spiny lobster aquaculture relies entirely on naturally settling seed lobsters (pueruli), resources of such seed are abundant in some areas, including Vietnam and the southern coastline of Indonesia (Priyambodo et al 2020). Indonesia can potentially be a major lobster aquaculture producer, providing government policy support such development and various constraints are addressed. In addition to defining and extending suitable production technology to farmers, a significant constraint is disease.

Cultured lobsters are susceptible to a range of diseases, including red body, black gill, milky hemolymph disease (MHD), red tail, and White-spot Syndrome Virus (WSSV) (Jones 2015; Sudewi et al 2020; Sukenda et al 2024). Strategies to manage diseases and improve health status include optimizing nutrition, providing optimal environment, and applying probiotics. Considerable research has been done to control disease and increase the growth performance of aquatic species by applying probiotics and symbiotic microbes (Nguyen et al 2014; Chauhan & Singh 2019; Khanjani et al 2024). An example involving spiny lobsters is using a combination of *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 elicited a beneficial response in lobster phyllosoma larvae (*Panulirus ornatus* (Fabricius, 1798)) infected with *Vibrio owensii* DY05 (Goulden et al 2012). In general, probiotics not only act to enhance the immune system but also help protect from pathogenic microbes by increasing intestinal mucus through integration with epithelial cells on the intestinal wall.

As invertebrates, lobsters do not have an adaptive immune response involving immunoglobulin (Young & Söderhäll 2002; Huang et al 2020), and only a natural, innate immunity. To increase the survival, growth, and health status of lobsters using dietary probiotics, research was performed to assess the stimulation effect on the digestive system and the protective effect on specific diseases. The function of an effective probiotic is to improve growth performance, stimulate nonspecific immunity, and protect from microbial pathogens by producing bacteriocin compounds that demonstrate bacteriocin-like activities (Daniels et al 2013).

The Research done by Leyva-Madrigal et al (2011) has demonstrated that the application of probiotics could improve growth performance, increase digestive enzyme resistance to diseases, boost immune gene expression, and reduce the prevalence of viral infections in white shrimp, *Penaeus vannamei* Boone, 1931. Similarly, the result of vibrio challenge tests in *Homarus americanus* showed a significantly higher transcriptional response of anti-lipopolysaccharide factors with increased expression of general immunity (Clark et al 2013; Nguyen 2023).

The use of probiotics in aquatic animal culture has demonstrated multiple benefits, including improvement of water quality, control of pathogenic bacteria and their virulence, avoidance of the use of antibiotics and consequentially reduced risk of antibiotic resistance, stimulation of the immune system, increased intestinal flora, reduction in the incidence of diseases and improved assimilation of diet (Haryanti et al 2021; Lein et al 2022; Pascual et al 2022; Chen et al 2023). Preliminary research results at the Institute for Mariculture Research and Fisheries Extension (IMRAFE) have shown that the application of yeast S*accharomyces cerevisiae* and a combination of *Bacillus cereus* BC and *Alteromonas* sp BY-9 added to a moist pellet diet, as compared with a control without probiotic, generated survival of 85.1, 85.9 and 81.8% respectively. Adding *S. cerevisiae* to the lobster diet significantly boosted immunity-related gene expression. The ProPO gene expression increased from 14 to 18, while the genes ALFHa-1**,** ALFHa-2, and ALFHa-4 showed an overexpression from 62.5, 50.6, to 94.5, respectively, after exposure to *Vibrio harveyi*, compared to the control group. Therefore, this study aims to explore the potential bacteria as candidates for probiotics for application to the rearing of spiny lobsters is strongly justified. Such application will be assessed regarding increasing survival, growth, and health status and may contribute to commercial applications to increase the production of lobster from aquaculture.

Material and Method

Sample collection. Seven individual wild lobsters were collected from the coastal waters of Siwi, Jembrana district of Bali. Average body weight, total length, and carapace length were 154.84±12.27g, 18.23±0.58 cm, and 7.97±0.14 cm, respectively. Bacteria were collected from the intestine by crushing 0.3 to 0.5 g of intestinal tissue in a Phosphate Buffer Saline (PBS) solution, which was then filtered to 0.45µm. The bacteria were cultured in Petri dishes on Marine 2216 agar (Difco, Cat No 212185, USA). After 24 hours of incubation at a temperature of 25^{o}C, bacterial colonies were selected based on different shapes, colors, and sizes. These bacteria were purified 3 to 4 times through sequential isolation onto new agar plates until pure, single colonies were obtained. All bacterial isolates were screened on Thiosulfate Citrate Bile Salt Sucrose-TCBS Agar (Difco Cat No. 265020, USA) to eliminate *Vibrio* sp. contamination. Further, the culture of the bacteria was prepared in tilted test tubes in preparation for future testing.

Identification of bacterial isolates as probiotic candidates. The identification of candidate probiotics was initiated by DNA extraction. Each isolate was analyzed using a Presto^{TM} Mini gDNA bacteria kit (Geneaid, Cat. No. GBB100. Taiwan), and the screening of DNA polymorphism was applied for amplification with PCR (Biometra 050-552, Thermocycler T personal Combi, Germany) using universal Primers AAM2. The nucleotide sequence of this primer is 2AAM2 (5'-CTG CGA CCC AGA GCG G -3'). Following PCR amplification, the PCR products were analyzed using electrophoresis. This screening was used to select the DNA fragmentation performance of each probiotic candidate isolate. Bacterial isolates with the same diversity of DNA fragments were identified as singular isolates. Preparation of samples from selected bacteria for further sequencing was done by PCR amplification of 16 SrRNA with pairs of primers 27F (5'-AGA GTT TGA TC (AC) TGG CTC AG-3') and 1492R (5'- ACGG (CT) TAC CTT GTT ACG ACT T-3') with target region of 1400 bp. The resulting nucleotide sequences were analyzed and edited using AB-PRISM (1st Base Singapore).

Characterization of candidate probiotics. Enzymatic hydrolysis activity testing included Lipase, Galatinase, Amylase, Urease, Casease, Lecithinase, and Chitinase for probiotic bacteria candidates. Isolated bacteria were subject to enzymatic testing through Extra Cellular Product (ECP) analysis using the cellophane plate method. Sterile cellophane was placed on the surface of Marine 2216 Agar (Difco Cat No 212185, USA) in a Petri dish, then inoculated with the selected bacteria and incubated for 24 hours at 25^{o}C. ECP from the surface of the cellophane was washed with PBS buffer solution at pH 7.0. The resulting suspension was centrifuged (Eppendorf 5415R, Germany) for 10 minutes at a speed of 13,000 rpm and a temperature of 4^{o}C. The supernatant was filtered through a membrane filter at 0.22 µm. Enzymatic activity for gelatinase, casease, amylase, lecithinase, lipase, urease, and chitinase was tested using a slider tube. Marine 2216 agar (Difco Cat No 212185, USA) media 2 % was prepared containing 0.4% gelatin, 0.4% casein, 0.2% starch, 0.25% yolk egg, 1% Tween 80.2% Urea and 2% phenol red to test the activity of the enzymes. The test involved placing a polyethylene tube on each agar plate filled with 200 µL filtrate of the ECP. The proportion of clear zone in the tube was used as a measure of the activity of the enzyme product for each candidate bacteria. The greater the extent of the clear zone indicated, the greater the hydrolysis by the enzymes in the agar substrate for each nutrient.

Culture of bacterial isolates as probiotic candidates. Isolated bacteria with a positive ability to enzymatically hydrolyze protein or carbohydrate were cultured in Marine 2216 Broth until they attained a peak density of 10^{9-10} CFU mL^{-1}. The culture media were sterilized by autoclave (Hirayama.HL-36Ae, Japan) at the temperature of 121^{o}C for 15 minutes. As aerobic bacteria require oxygen, aeration was provided to accelerate growth. The duration of incubation was 48 hours. Assessment of growth form was made for each candidate bacteria using 300 mL flask glass with aeration.

Observations were done by UV-VIS spectrophotometer (UV mini 1240, Shimadzu, Japan) with OD 630 nm.

Production of moist pellet diet for testing of probiotics. A moist pellet was prepared using a previously tested formulation (Giri et al 2023) that supported good survival and growth of cultured *P. homarus* (Irvin & Shanks 2015). The diet included fish and krill meals, fresh shrimp and squid, Carboxymethyl Cellulose (CMC), and vitamin mix. Four bacterial isolates with putative probiotic characteristics were combined and 100 ml of the bacterial culture was used per 1 kg of moist pellet mix, to provide a density of probiotics equal to 10^5 CFU mL^{-1}. The moist pellet was air-dried for 3 hours and kept in a refrigerator at a temperature of around 6°C.

Proximate analyses of all feeds were carried out following Giri et al (2023), Moisture was analyzed by drying samples of feed at 110°C in an oven (Memmert 854, Germany) until a constant weight was obtained. Crude protein was determined according to the Kjeldahl procedure (using Kjeltec TM 8100, Foss). Total lipid was extracted using chloroform and methanol. Ash was analyzed using a muffle furnace (Carbolite ESF S20, England) at 550°C. The proximate compositions of all feeds used in this experiment are presented in Table 1.

Table 1

Proximate composition of moist diet used for rearing of spiny lobster, *P. homarus*

Proximate composition (%)	*Moist diets without probiotics*	*Moist diets supplemented with combination of probiotic*
Ash	14	13.56
Moisture	2.49	2.81
Lipid	7.24	6.17
Proteins	50.03	52.05
Fibre	16.58	16.59

Use of probiotics fed by a moist pellet in the rearing of P. homarus. Four bacterial isolates were identified in the assessment of lobster intestinal bacteria as having potential probiotic capacity. These bacteria were combined for testing as a dietary supplement in the performance of *P. homarus* lobsters under culture conditions. Two treatments were applied to a feeding trial using the moist pellet defined above with a supplemental probiotic (diet A) and without the probiotic (diet B). The initial mean body weight of lobsters used in the experiment was 70.34±12.03g. The lobsters were reared in 4 m^3 capacity concrete tanks filled with seawater to a depth of 60 cm, a density of 15 individuals $m^{-3,}$ and fed two times a day (am and pm) for three months. Clean, filtered seawater was supplied on a flow-through basis, and uneaten food and debris were siphoned off each morning. Strict biosecurity was applied, especially cleaning of all equipment used and the hands and footwear of technicians attending. Concrete blocks with holes and PVC pipes were provided as shelter. At the end of the experiment, ten lobsters were challenged with MHD through muscle injection. The number of hemocytes was calculated after the challenge and the immunity level gauged through gene expression was determined by RT-qPCR (AB Applied Biosystem 7500 Fast Real-Time PCR System, USA).

Hemocyte count. Hemolymph was collected from 10 lobsters through the ventral sinus cavity using a 25 gauge needle and 3 mL syringe containing an anticoagulant compound (2% NaCl, 0.1 M glucose, 30 mM Na citrate, 26 mM citric acid, 10 mM EDTA) with lobsters held on ice. Hemolymphs were collected in this manner each month from five individuals for hemocyte counts. An amount of 10 µL of hemolymph was used to calculate the total hemocyte using a hemocytometer under a binocular microscope (Nikon SMZ1000, Japan) with 400x magnification.

Immunity Status. Immunity status was estimated by comparing mRNA expression levels in the hemolymph throughout the experiment. Gene expression-related immunity was analyzed by RT-qPCR (AB Applied Biosystem 7500 Fast Real-Time PCR System, USA). The immune response was observed by a challenge test with milky hemolymph Diseases - MHD through muscle injection (100 µL each lobster). Challenge tests were done for 96 hours, with hemolymph collected every 24 hours.

Gene expression-related immunity

Isolation RNA and cDNA synthesis. The hemolymph collected was then centrifuged at the speed of 12,000 rpm at a temperature of 4°C for 10 minutes. The solidified hemolymph was then washed once in a cold anticoagulant solution. Afterward, total RNA was extracted using a lysis RNA extraction solution followed by the IQ-2000 (Code 38220019, Taiwan) method. Synthesis of cDNA (complementary DNA) was performed using Agilent Affinity Script qPCR cDNA Synthesis kit (Cat ≠600559, USA). A 20 µL volume was used consisting of 10 µL first strand master mix (2x), 3 µL oligo (dT) primer, 1.0 µL affinity script RT, and 3 µg total RNA. Solutions in microtubes were incubated at a temperature of 25°C for 5 minutes, 42°C for 15 minutes, and 95°C for 5 minutes, respectively. The cDNA was kept on ice to stop synthesis and then kept in a freezer at -20°C for further analyses.

RT-qPCR analyses of gene target related to immunity of spiny lobster P. homarus. A profile of immune status was determined by Gene Expression Transcription using RT-qPCR (AB Applied Biosystem 7500 Fast Real-Time PCR System, USA) with a specific primer to provide a quantitative measure of immunity following the methods of Wang et al (2010) and Clark et al (2013). Analysis of Prophenol Oxydase ProPO, Glutathione Peroxidase (GPx) using Clotting Protein (CP) followed the method of Wang et al (2010), while analysis of ALFHa-2 followed the method of (Clark et al 2013) with an internal control using 18SrRNA (Table 2). Analyses of RT-qPCR were carried out using the ABI Prism-7500 system, and the detection sequence by 5x Hot Fire pol Eva-green qPCR mix (ROX). The volume of reaction for amplification of cDNA was 20 µL with final concentration 1x hot Master mix (Rox), Primer F/R 10 pmol of each 250 nM, NFW (Nuclease Free Water) was added until the volume reached 20 µL and cDNA (0.01 ng μL^{-1}). The temperature cycling for RT-qPCR consisted of holding temperatures of 50°C for 2 minutes, an initial temperature for denaturation of 95°C for 15 minutes followed by 95°C for 15 seconds, and an annealing temperature of 60°C for 30 seconds with an extension temperature of 72°C for 20 seconds. The thermal cycle was repeated for 40 cycles. Calculation of ΔCt from the threshold cycle PCR (Ct) gene was tested and normalized by relative value to Ct 18sRNA (internal control) in the same sample. The value of ΔΔCt was calculated from ΔCt of the tested sample - ΔCt (initial expression). The representative relative multiple which was different from the initial expression, was calculated with $2^{-\Delta\Delta Ct}$.

Table 2

The sequence of primers that were used to determine the immune system-related gene expression by RT-qPCR analysis of lobster *P. homarus*

Immune System	*Target gen*	*Name*	*Primer*	*Sequence (5"-3")*	*Gen Bank*
proPO activating system	prophenoloxidase	proPO	proPO-F/R	F:GAGATCGCAAGGGAGAACTG R:CGTCAGTGAAGTCGAGACCA	EF 565469
Antimicrobial peptide system	Clotting protein	CP	CP-F/R	F:TCTTTGCGCAGTTGGTGATC R:TGAGGTGACCGAGTGCAAAA	DQ984182
Antioxidant defense mechanism	glutathione peroxidase	GPx	GPx-F/R	F:TTTTTCCGTGCAAAAAGGAC R:TAATACGCGATGCCCCTAAC	AY 973252
Anti lipopolysaccharide	Anti lipopolysaccharide	ALF-2	ALF-2 F/R	F:AGACTACCACTGACTTCGTGAGGA R:TCTCGGGATGATCCGTTAACACCT	FC556430
Internal control	18s RNA	18s	18s-F/R	F:AGCAGGCTGGTTTTTGCTTA R:AGCAGGCTGGTTTTTGCTTA	AF 186250

Data analysis. The results of this study were expressed as means ± standard deviation. The research design of this study was conducted as an experimental design with a T-test. Analysis was performed to evaluate the potential effect of endogenous bacteria as probiotics on growth, survival, and immunity in spiny lobster cultures. The differences were considered statistically significant at a 95% confidence level (p<0.05).

Results

Sample collection and isolation of endogenous bacteria as probiotic candidates. The results of bacteria collected from lobster intestinal tissue were characterized by white color on Marine 2216 Agar (Difco Cat No 212185, USA) and green and yellow color on TCBS agar (Difco Cat. No 265020). Bacterial counts are presented in Table 3. Purification results showed 94 pure isolates grew in Marine 2216 Agar (Difco Cat No 212185, USA) and 16 isolates (*Vibrio* sp.) grew in TCBS agar (Difco Cat. No 265020).

The results of observations on the total number of bacteria isolated from 7 lobsters caught from the wild showed differences in bacterial populations (P<0.05) in lobster samples no. 1, 2, and 7 compared to lobster samples 3 and 5 as well as for lobster samples 4 and 6. The population of *Vibrio* sp. bacteria in the lobster samples showed a bacterial population with green color and yellow colonies in 4 lobsters, while in 2 lobster samples, the colonies were only yellow.

Table 3

Result of bacterial isolation from 7 spiny lobsters, *P. homarus*

Lobster	*Body weight (g)*	*Total length (cm)*	*Carapace length (cm)*	*Total bacteria CFU mL^{-1}*	*Total Vibrio* sp. *CFU mL^{-1}*
1	165.2	18.0	7.8	$137\pm2.45 \times 10^{4a}$	$111\pm9.3\times10^{2}$ (Y)^{a}
2	134.3	17.2	8.0	$2\pm0.5 \times 10^{6\ a}$	$54\pm4.6\times10^{2}$ (Y)^{b}
3	154.8	18.2	7.8	$10\pm1.3 \times 10^{6\ b}$	Uncountable
4	155.7	18.0	8.0	$8\pm1.75 \times 10^{4\ c}$	$49\pm5,1\times10^{2}$ (G)^{a} $3\pm1.7\times10^{2}$ (Y)^{c}
5	170.3	18.5	8.0	$6\pm2.1\times10^{6\ b}$	$6\pm2.7\times 10^{2}$ (G)^{b} $20\pm3.2\times10^{2}$ (Y)^{c}
6	159.4	18.7	8.2	$3\pm0.75\times10^{4\ c}$	$4\pm1.5 \times 10^{2}$ (G)^{b} $45\pm3.5\times 10^{2}$ (Y)^{b}
7	144.2	19.0	8.0	$1\pm0.2 \times10^{4\ a}$	$4\pm1.3 \times 10^{2}$ (G)^{b} $89\pm6.3\times 10^{2}$ (Y)^{a}

Remarks: G: Colony of *Vibrio* with green color; Y: Colony of *Vibrio* with yellow color

Identification of endogenous bacteria as probiotic candidates. The results of screening bacteria collected from the lobster gastro-intestine using RAPD (Randomized Amplification Polymorphism DNA) with the 2 AAM2 primer method were, that out of 94 isolates, only 16 isolates showed a different pattern of DNA fragments (Figure 1).

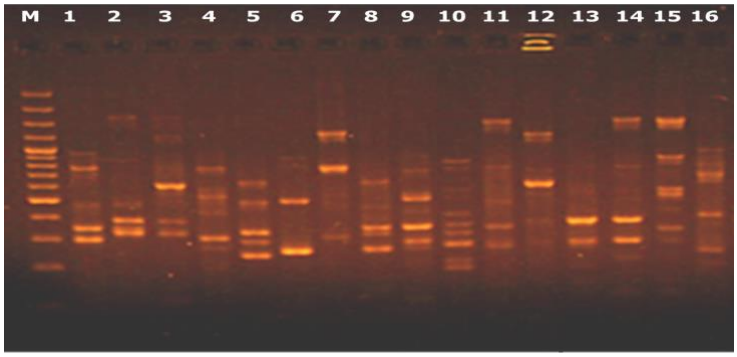

Figure 1. The pattern of DNA fragmentation of bacterial isolates from intestinal tissue of spiny lobster *P. homarus* using universal primer 2 AAM2.

Based on the analyses performed, of 16 isolates, only 4 isolates could hydrolyze enzymatically (Extra Cellular Product) carbohydrate, protein, and fat. Results of the Blast-N showed high similarity (97 to 98%) between analyzed samples for DNA sequence for each accession. The bacteria with potential as probiotics were identified as *Photobacteria damselae* N-5*, Bacillus subtilis* C-1*, Bacillus oceanisediminis* H-3*,* and *Bacillus amyloliquefaciens* I-5. The DNA sequence of the bacterial strains is similar in Table 4.

Table 4

Similarity of DNA sequence isolated from bacteria of probiotic candidates compared with total gene sequence on geneBank from each accession number

Species Name	*Code*	*Forward*	*Similarity (%)*	*Accession*	*Reverse*	*Similarity (%)*	*Accession*
Phorobacterium damselae	N-5	F	97	KU245711.1	R	98	KC275152.1
Bacillus amyloliquefaciens	I-5	F	97	KF836515.1	R	97	HQ844481.1
Bacillus subtilis	C-1	F	98	KP735610.1	R	97	KC456632.1
Bacillus oceanisediminis	H3	F	97	KP196809.1	R	97	JF411235.1

Hydrolyses enzymatic Activity tests of probiotic candidates*.* Based on the results of the enzymatic tests, 4 bacterial isolates could hydrolyze enzymatically. Results of enzymatic hydrolysis analyses are presented in Table 5. The result suggests that all isolates were able to hydrolyze Gelatinase, Casease, Amylase, Lecithinase, and Lipase but not Chitinase, while isolate C-1 did not hydrolyze urease.

Table 5

Enzymatic activity of endogenous bacteria as candidates of probiotics isolated from the intestine of spiny lobster *P. homarus*

Code of probiotic isolate	*Gelatinase (Gelatin)*	*Casease (Casein)*	*Amylase (Starch)*	*Lecithinase (Yolk egg)*	*Lipase (Tween 80)*	*Urease (Urea)*	*Chitinase (Phenol red)*
P. damselae N-5	+ (24-96 H)	+ (48-96 H)	+ (24-96 H)	+ (24-72H)	+ (24-96H)	+ (48-96H)	- (24-96 H)
B. amyloliquefaciens C-1	+ (96 H)	+ (48-96 H)	+ (24-96 H)	+ (96H)	+ (48-96H)	- (24-96H)	- (24-96 H)
B. subtilis H-3	+ (24-96 H)	+ (48-96 H)	+ (24-96 H)	+ (96H)	+ (24-96H)	+ (48-96H)	- (24-96 H)
B. oceanisediminis I-5	+ (96H)	+ (48-96 H)	+ (24-96H)	+ (96H)	+ (48-96H)	+ (24-96H)	- (24-96H)

Culture of endogenous bacteria as candidates of probiotics. The bacterial strains identified as probiotic candidates were cultured in a flask glass in a 300 mL volume of Marine 2216 Broth (Difco cat. no.279110, USA) with aeration to supply oxygen for 96 hours (4 days) with two replications. The optical densities were measured by a UV-VIS spectrophotometer (UV mini 1240, Shimadzu, Japan) with an Optical Density of 630 nm. The results are shown in Figure 2. The growth of isolates *Bacillus amyloliquefaciens* I-5 and *Bacillus subtili* C-1 was slightly slower than isolates No-5 and H-3, although all isolates reached similar densities by day 3. This indicates that the growth of bacterial isolates *Photobacteria damselae* N-5 and *Bacillus oceanisediminis* H-3 was significantly different ($P<0.05$) compared to strains I-5 and C-1.

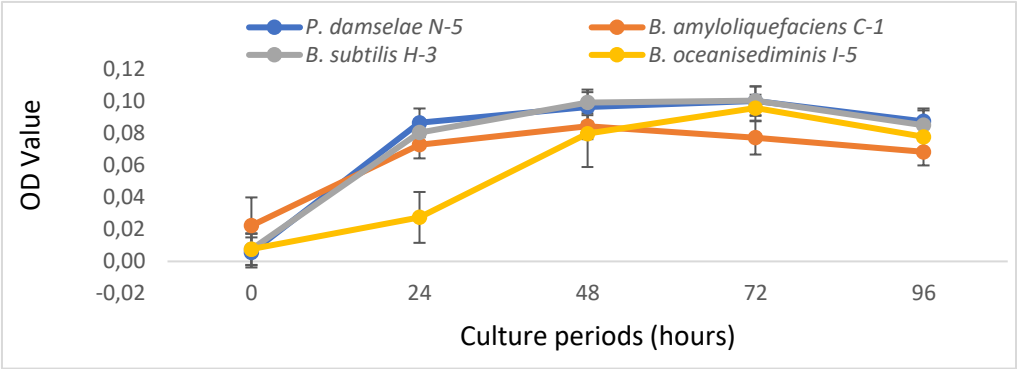

Figure 2. Cell density from four bacterial isolates from spiny lobster, *P. homarus* as probiotic candidates was measured by spectrophotometer with an Optical Density of 620 nm.

Assessment of probiotics as a dietary supplement in the rearing of spiny lobster *P. homarus.* Application of the probiotic mixture as a dietary supplement significantly positively impacted growth in the rearing of spiny lobster *P. homarus* ($P<0.05$), as shown in Figure 3. The final mean body weight of lobsters fed with the probiotic supplement (A) was 198.21±9.72 g, some 17% larger than those fed without the probiotic (B) at 169.76±14.51 g. Although the survival of lobsters fed the probiotic-supplemented diet was higher than those fed without the probiotic, the difference was not significant. It is worth noting that the probiotic-supplemented diet had a higher protein content (52.05%) than the diet without (50.03%) (Table 1). However, the small difference is unlikely to fully explain the difference in growth. It is more likely that the probiotic had a direct positive impact on growth. Rivaie et al (2023) showed that the result of research using various types of feed for *P. homarus* juveniles, namely fish pellets, shrimp pellets, and flesh mussels, provide different results on growth performance, physiological responses, and behavior changes of juvenile spiny lobsters. Appropriate pellet formulation is recommended to improve the growth performance of *P. homarus* juveniles.

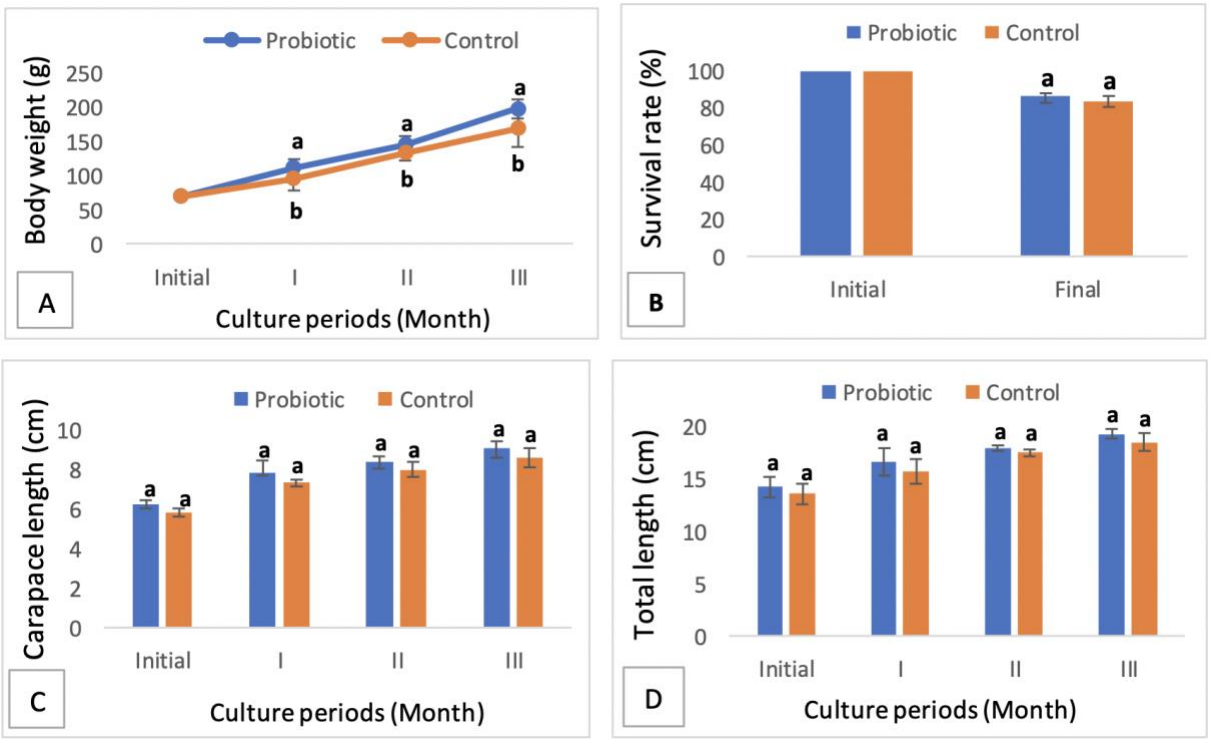

Figure 3. Growth of weight (A), survival rate (B), growth of carapace length (C), and growth of total body length (D) of spiny lobster *P. homarus* reared with and without supplementary probiotics.

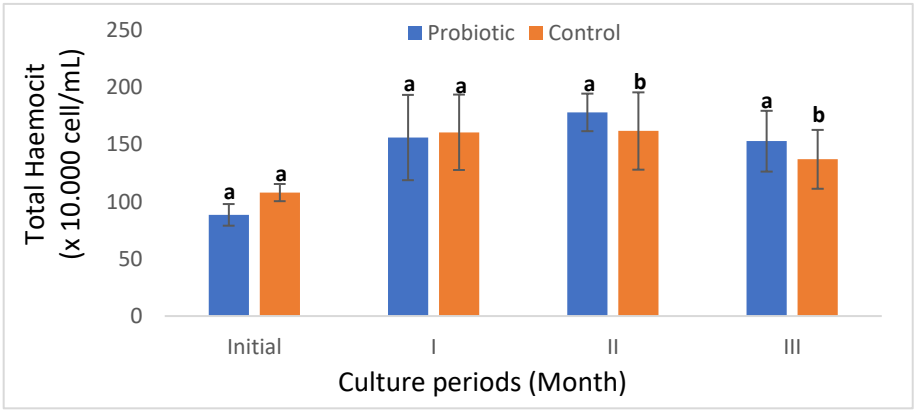

Figure 4. Hemocyte cell count for spiny lobster *P. homarus* reared with and without (control) supplementary probiotics.

Examination of the total hemocyte count of spiny lobsters fed a diet with and without probiotics, showed a positive impact at two and three months of culture. The total hemocyte count for lobsters fed with the diet containing probiotics was higher than the control without probiotics. Total hemocyte count is a strong indicator of lobster immunity.

Immunity status: gene expression-related immunity in spiny lobster P. homarus. Challenging lobsters fed diets with and without probiotics, with MHD, showed a stronger immune for those fed with the probiotic. Dietary supplementation with a combination of probiotics increases the immunity expressed on ALF2 genes by 18.74±0.63 and 13.58±0.3 fold after exposure time of 24 and 72 hours, while on ProPO genes provides a multiple of the expression value of 13.61±0.22 fold after challenge of 48 hours. The expression value of CP genes provides 35.41±0.45 and 31.01±0.45 fold, after 24 and 48 hours challenge, respectively, compared with the control. Expression of the GPx gene after a 48-hour challenge with MHD was increased by 94.74±0.20 fold. The findings demonstrate that the application of probiotics may increase the immunity of spiny lobsters.

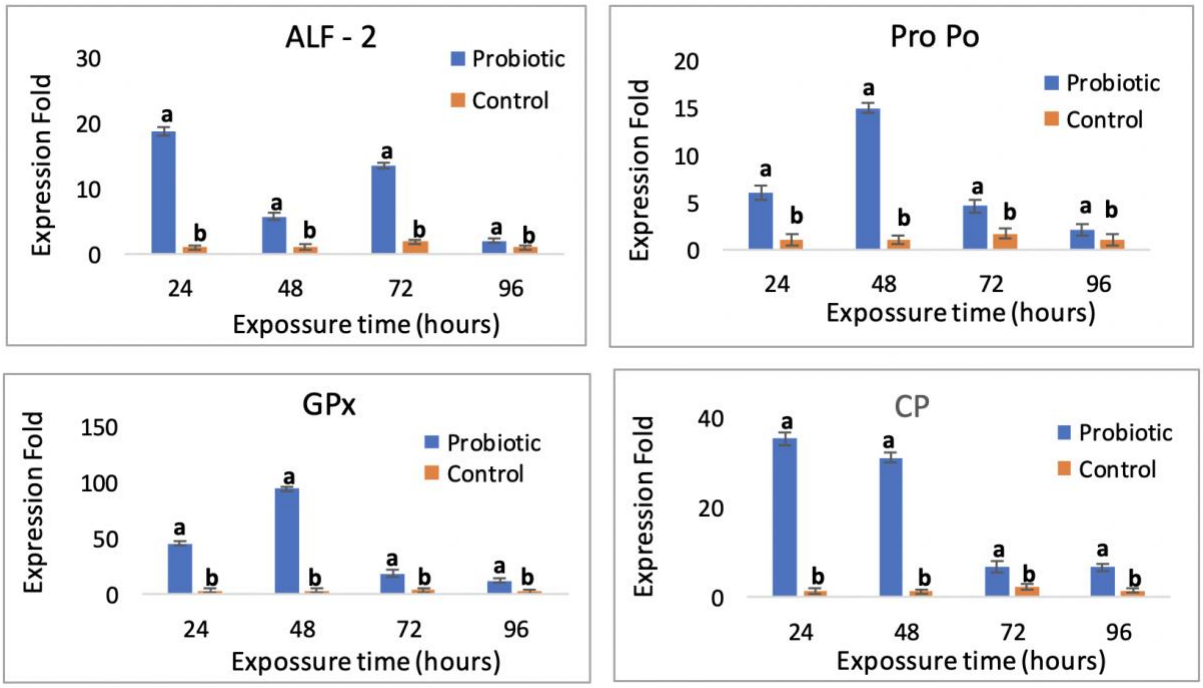

Figure 5. Quantitative relative mRNA of anti-lipopolysaccharide factor (ALFHa-2), ProPO, CP and GPx of spiny lobster *P. homarus* reared with combination probiotic after MHD challenge test.

Discussion. Bacterial communities, especially from the gastrointestinal tract of aquatic animals, are widely known to have an important role and contribute to the growth or health performance of animal hosts (Amin et al 2024). Therefore, the results of the exploration of bacteria originating from the gastro intestine of spiny lobsters collected from nature are potential sources to discover novel probiotics for aquaculture purposes. Oelschlaeger (2010) states that among the modes of action of probiotics, they can modulate host intestinal defenses in both innate and acquired immune systems, and this action is very important in preventing and treating disease infections and inflammation of the digestive system. Research results of Jayakumar et al (2011) demonstrated that dietary protein content had a significant impact on the growth of juvenile *P. homarus* lobsters. This research applied feed formulation with relatively high protein level differences (54.9%, 45.9%, and 35.88%). The results indicated that formulated pellet feed with relatively higher protein levels (54.9%) increased growth and total production of lobsters. Research on the use of fish and shrimp hydrolysate in lobster feed formulations shows a tendency to increase the growth, survival, and molting frequency of spiny lobsters (Astuti et al 2022). Studies by Wee et al (2024) and Daniels et al (2013) explored the effects of combining probiotics and prebiotics (synbiotics), demonstrating specific changes in the composition of microflora activity in the gastrointestinal system, which provided benefits for lobster health. This synergistic action was shown with the combination of *Bacillus* spp. and mannan oligosaccharide, which improved the survival and growth of *Hommarus gammarus* (Linnaeus, 1758) larvae. Foysal et al (2020) state that two bacteria *Lactobacillus acidophilus* and *Lactobacillus plantarum* have great potential for health as well as gut microbial composition and interaction network between gut microbiota and immune system in crayfish *Cherax cainii* Austin and Ryan 2002.

Testing using RT-qPCR helps understand the transcription of genes during the MHD challenge test. The expression level for RNA gene individually was normalized by 18s rRNA as reference for endogenous (internal control) expressed at 0 hours. The results demonstrate a positive impact of probiotics on the lobster's immune defense. This was evidenced by the activation of ALF-2 (anti-lipopolysaccharide factor), which significantly increased the number of lobsters fed the combination probiotic. ALF plays a role in neutralizing lipopolysaccharide (LPS), minimizing fungus infection and viral growth (Wang et al 2012). The activation of ProPO in crustaceans is thought to be an important aspect of innate immunity, particularly as a catalyst for melanization in invertebrates. Wang et al 2010 showed that the expression level of ProPO increased in *Penaeus chinensis* (Osbeck, 1765), which were infected with *Vibrio anguillarum*. Enhancement of proPO is important as a response to *Vibrio* spp. infection. The activation process of the ProPO system requires the participation of proteases, requiring control accuracy to prevent tissue damage. Phenoloxidase is produced by activation of prophenoloxidase (ProPO system) through a phenomenon known as "Cascade" using various methods. The immune system of lobsters, as in other invertebrates, depends on innate immunity. Innate immunity is divided into humoral defense (activation of proteolytic substances such as prophenoloxidase/ProPO system, mechanisms for clotting hemolymph, melanization responses, and antimicrobial responses) and cellular defense (phagocytosis, cellular degranulation, and release back or factor defense). The innate immunity in invertebrates is mediated by hemocytes within the blood circulation.

In injured lobsters, the blood clotting system plays an important role in providing very fast clotting to avoid a loss of hemolymph. This fast-clotting system also aids in the prevention of pathogens from ingress into the hemocoel. The clotting system and anti-microbial peptide system play a role in the mediation of hemolymph coagulation by triggering the polymerization of the clotting protein (CP). RNA interference was used to show that CP plays a role in defense against bacteria (*V. penaecida*) and the WSSV virus in kuruma prawn (*Marsupenaeus japonicus*) (Maningas et al 2008). Glutathione peroxidase (GPx) is an antioxidant enzyme that mediates damage caused by oxidative processes. GPx transforms hydrogen peroxide into water and oxygen with a catalyzation process. When lobsters are infected by any kind of disease, they will be subject to oxidative stress.

The response of immune-related genes in lobsters to bacterial disease infection will help to explain the immune response of lobsters to disease. The use of RT-qPCR for the characterization of changes in mRNA expression from 4 immune-related genes after challenges with MHD was able to provide a clear functional role of probiotics in the enhancement of the immune response. The results confirm that the application of probiotics is an effective method for preventing MHD infection through the enhancement of lobster immunity. This research is currently limited to a laboratory scale. In the future, it needs to be scaled up to implement lobster culture at the farmer level.

Conclusions*.* Based on the present findings, *Photobacterium damsela* N-5*, Bacillus subtilis* C-1, *Bacillus oceanisediminis* H-3, and *Bacillus amyloliquefaciens* I-5 could be used in combination as an effective probiotic. By adding this probiotic to the diet of spiny lobsters, the immunity level significantly improves after challenging MHD. These findings can be applied in lobster aquaculture to improve survival, growth, and overall health. The results confirm the application of probiotics could be an effective method for preventing MHD infection through the enhancement of immunity in lobsters, but it requires further studies.

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Conflict of interest. The authors declare that there is no conflict of interest.

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