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The effect of supplementation of *Lactococcus lactis* strain as probiotic on the growth and survival of *Litopenaeus vannamei*

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Abstract

Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) is a bacterial disease in shrimps caused by pathogen *Vibrio parahaemolyticus*. It has culminated in huge loss in global shrimp production due to mass mortality. A probiotic strain *Lactococcus lactis* strain FA1 was recently isolated from shark intestine, showing inhibition towards the growth of the pathogen. Due to inhibitory potential, the effect of probiotics strain on growth performance of shrimp infected with *V. parahaemolyticus* was evaluated. The probiotic strain was incorporated into feed for juvenile shrimps *Litopanaeus vannamei* for 3 weeks before which they were then challenged with pathogen *Vibro parahaemolyticus*. The study compares 4 shrimp groups: Control (Without any treatment); Group A (Probiotic treated, uninfected); Group B (Probiotic treated, infected) and Group C (No probiotic, infected). The survival and growth performance (weight and length gain) of shrimps were evaluated in the following 30 days. Statistical analyses (ANOVA; Post Hoc Tukey) were used to compare between shrimp groups. In general, infected shrimp demonstrated some of the key symptoms of AHPND (pale or white hepatopancreas), transparent body and erratic swimming behaviour. The supplementation of probiotics resulted in an improved survivability (65±1%) compared to infected shrimp (45±1%). The probiotic treated shrimp group showed to have better % body weight gain, in which weight gain between group B (treated/infected) and group C (untreated/infected) were significantly different (at P=0.046); and so, to group A (treated/uninfected) when compared with group C (P=0.047). There is however no significant difference in % length gain between the groups. Meanwhile, some of the infected shrimps were able to show recovery from the infection. Pre-infection probiotic treatment showed better performance compared to post-infection probiotic treatment. This indicated that the strain *Lactococcus lactis* is highly suitable for use as the future probiotic in shrimp aquaculture.

Keywords: acute hepatopancreatic necrosis disease; early mortality syndromes; *Lactococcus lactis*; *Litopenaeus vannamei*; shrimp probiotics

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Introduction

From year 2009, the intensive and productive shrimp farming around Asia has begun to be affected by an emerging outbreak called early mortality syndrome (EMS) or more descriptively called acute hepatopancreatic necrosis syndrome (AHPNS) (De Schryver *et al.,* 2014). This disease was later recognised to be caused by a toxin producing rod-shaped Gram-negative bacterium *Vibrio parahaemolyticus.* The infected shrimp normally shows symptoms with white or pale colouration of hepatopancreas, shrunken or reduction in the size of the organ (atrophy) and 100% shrimp in motility (Chiew *et al.,* 2019). Shrimp production losses which initially sparked in South of China has later spread to other countries in the Southeast Asia, culminating in new form of shrimp pandemic. This outbreak has resulted in the collapse in shrimp industrial network which have huge social and commercial impact. It is likely that this outbreak will emerge again in the near future (Kua *et al.,* 2016).

Antimicrobial agents such as chemical disinfectant, fungicide and antibiotics, has been the method of choice for farmers in managing the situation during outbreak. Unfortunately, the uses of these chemicals are poorly regulated. The excessive and unregulated use of antibiotic have resulted in the emergence of new resistant strain, a phenomenon which have been so common in other sectors such as food and health (Preena *et al.,* 2020). This is exacerbated by the discharge of aquaculture waste from cultivation pond, the perfect source for the release of antibiotic resistance microbes into the environment. Thus, the poor aquaculture waste management has now contributed to another public health concern. Meanwhile, the presence of antibiotic residues in animal tissues destined for human consumption have far reaching consequences. The presence of chemical residue in exported shrimp has resulted also in export ban, jeopardising the economy of the producing countries. For instance, USFDA in 2006 has banned shrimp product from Malaysia due to antibiotic contamination (Times, 2016). Concern about antibiotic-resistant microorganisms has led to suggestions of alternative disease prevention methods, including the use of microorganisms as biocontrol agents. Probiotics are microorganisms that are associated and brings beneficial effects to the host. Probiotics are able to eliminate pathogenic bacteria, improve or enhance the immune system, and restore the gut balance of microflora of the host (Anee *et al.,* 2021). As much as it was in human or animals, probiotics could render similar benefits in aquatic species (Amenyogbe *et al.,* 2020). Therefore, the application of probiotics as a form of eco-friendly supplement in aquaculture to improve the physiology, growth performance, and immune responses in fish have attracted great attentions.

Probiotic strains generally belong to gram positive lactic acid bacteria (LAB) (Abedi and Hashemi, 2020), which normally able to ferment food material to produce lactic acid, forming the basis of food fermentation and preservation. Lactococcus lactis is a gram-positive coccoid lactic acid bacterium (LAB) that was initially discovered from dairy environment. Now, the use of this bacterium was made broadened beyond just in dairy industries; into genetic engineering and microbial cell factory (Zielińska and Kolożyn-Krajewska, 2018). In our previous work, a few strains of Lactococcus lactis strains were successfully isolated from the intestine of Black tip shark (Hamid *et al.,* 2020). These novel strains were able to inhibit *Vibrio parahaemolyticus,* the pathogenic strain which has elicited Early Mortality Syndrome (EMS) in shrimp. Therefore, *L. lactis* strain has potential to be used as biocontrol agents in shrimp aquaculture. This strain could offer several advantages compared to other strains currently in used. For instance, spore-forming *Bacillus* strains has been commonly used as shrimp probiotic (Golder *et al.,* 2022). However, the use of *Bacillus* strain is of great concern especially in food product since this strain is a spore former. Despite of some of the *Bacillus* strain are still not recognised as Generally Regarded as Safe (GRAS), making consumer acceptance for this strain is still a challenge (Elshaghabee *et al.,* 2017). Since *Lactococcus lactis* is LAB belonging to GRAS status, it is more readily accepted by consumers and not being subjected to food regulation (Cano-Lozano *et al.,* 2022). Meanwhile, there were studies attempting to use other *Vibrio* strains which are phylogenetically related to the common pathogen (Thompson *et al.,* 2010). Moreover, the horizontal gene transfer of multidrug resistance gene between Vibrio species is not that uncommon (Deng *et al.,* 2019). Thus, in view of these situations, *L. lactis* strains is highly suitable to be developed as a probiotic for shrimp. Nevertheless, there is still lack of report on the use of *L. lactis* strain in shrimp aquaculture.

Based on the antagonistic properties of the *L. lactis* strain observed towards pathogenic *V. parahaemolyticus*, this study serves to evaluate the effect of consuming this strain on the shrimp growth and performance. Juvenile shrimp *Litopenaeus vannamei* was fed with probiotic strain and later was subjected to challenge with the pathogen. The effect of probiotics supplement on the survivability, mortality, pathogenic symptoms, the length, and weight gains of the infected and uninfected shrimps were evaluated. This study statistically compares 4 shrimp groups exposed to different treatments. Our result indicated the *L. lactis* strain has future prospect to be used as probiotics for shrimp aquaculture, especially in managing EMS infection.

Materials and Methods

Bacterial strains

The two microbial strains used in this work; 1) the previously isolated *Lactococcus lactis* strain FA1 (Genbank: MN975529.1) used as probiotics strain for shrimp feeding (Hamid *et al.,* 2020); and 2) pathogenic strain *Vibrio parahaemolyticus* used to elicit EMS disease on shrimp. These strains were retrieved from bacterial stock collection available at Kulliyah of Science, International Islamic university Malaysia, Indera Mahkota Campus. These strains were subjected to microbial gram staining for morphological characterization.

Antimicrobial assay

Agar well diffusion method was used for inhibition study with modification (Fredua-Agyeman and Gaisford, 2019). From glycerol stocks, *L. lactis* strain was resuscitated on De Man, Rogosa and Sharpe (MRS) agar containing (per liter) 10 g Peptone, 5 g yeast extract, 10 g meat extract, 20 g glucose, 1.0 g tween 80, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g manganese sulphate, and 2.0 g disodium phosphate and 15 g agar. The strain was then propagated on MRS broth medium. The *V. parahaemolyticus* strain was revived on nutrient agar medium containing (per liter) of 0.3 g beef extract, 0.5 g peptone, 0.5 g NaCl, 15 g agar; and then inoculated to nutrient broth (NB). All media were incubated overnight at 30 °C, with agitation at 150 rpm until optical density (OD) had reached approximately 600 nm. *V. parahaemolyticus* broth suspension (~250 to 300 µL) was spread evenly on the surface nutrient agar (NA) plate. Wells (~5.2 mm diameter) were made on agar plate which then was filled with 50 µL suspension broth of *L. lactis* strain. The plates were incubated overnight at 37 °C and the inhibition zones were observed and measured after 24 hours incubation.

Probiotic shrimp feeding experiment

The probiotic strain *L. lactis* was inoculated on MRS broth, incubated overnight at 30 °C, at 150 rpm until reaching the cell density of approximately 1 x 10^8 CFU/ml (~0.5 McFarland) concentration which was used for shrimp feeding. This cell suspension was evenly sprayed on shrimp feed at 150 ml of cell suspension per 300 g shrimp feed. The shrimp feed was air dried and stored at 4 °C. Four plastic aquarium tanks (30 x 45 x 60 cm) were disinfected with 10% v/v sodium hypochlorite water for 4 hours, following rinsing with distilled water. The shrimps were divided into 4 different treatment groups, as shown in Table 1. Figure 1 shows the tanks used in this study which was conducted in duplicates. Each tank was filled with 13L filtered seawater and set up with full aeration, maintaining constant pH at 7.5 ±0.5 and temperature at 25±0.5 °C. There is no water exchange for the period of 51 days of the experiment, any water loss due to evaporation was compensated by adding filtered sea water. The tanks were filled with 20 juvenile white-legged shrimp *L. vannamei* obtained from a research station (Institute of Oceanography and Maritime studies, INOCEM) located at Kg Cherok Paloh, Kuantan.

Figure 1. The plastic tanks containing shrimps *Litopenaeus vannamei* groups employed in this work

Table 1. Shrimp groups and treatments

Groups	Control	Treatment A	Treatment B	Treatment C
Number of shrimps	20	20	20	20
Treated with probiotic	No	Yes	Yes	No
Infection with *V. parahaemolyticus*	No	No	Yes	Yes

Table shows four shrimp groups with different exposures to combination of treatment which is: Control (Without any treatment); Group A (Probiotic treated, without infection); Group B (Probiotic treated, with infection); and Group C (No probiotic, with infection).

Shrimp infection with V. parahaemolyticus

The shrimps *L. vannamei* was infected with pathogen by immersion techniques with modification (Hong *et al.,* 2016). The pathogenic strain *V. parahaemolyticus* was propagated into the nutrient broth medium, incubated overnight at 30 °C, at 150 rpm until the cell density reached approximately 1.5 x 10^8 CFU/ml. This cell suspension was used to infect shrimp after 3 weeks (21 days) of probiotics treatments. About 250 ml of *V. parahaemolyticus* bacterial suspension was poured into 750 ml of filtered seawater filled with juvenile shrimps. Shrimps were left in seawater containing bacteria suspension for about 7 hours. After 7 hours, the shrimps were rinsed and transferred to tanks filled with fresh seawater. Appearance of AHPND/EMS symptoms, and any changes in shrimp behaviour were observed for a week. In the following 30 days after the infection, the numbers of shrimp survivability or mortality were recorded. The average body weight and length of shrimps in each tank was recorded just before treatment, and after the treatment (at 30 days after infection). The shrimps were starved for 24 hours before each treatment. Shrimps were fed three times daily during the pre-infection stage (21 days), and during the following post infection stage (30 days). The average body weight and length of shrimps in each treatment was recorded. The shrimp body weight gain and change in body length were measured based on the following equations:

$$\text{Body weight gain (g)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}}$$

$$\text{Percentage of weight gain} = \text{Body weight gain x 100 (\%)}$$

$$\text{Body length change (cm)} = \frac{\text{Final length (cm)} - \text{Initial length (cm)}}{\text{Initial length (cm)}}$$

$$\text{Percentage of length change} = \text{Body length change x 100 (\%)}$$

The feed conversion ratio (FCR) is calculated based on following equation:

$$\text{FCR} = \frac{\text{Weight of feed consumed (g)}}{\text{Body weight gain (g)}}$$

Post-infection probiotic treatment

Another set of experiments was also carried out in which the probiotic treatment on shrimp was administered after infection exposure. All other parameters and experimental conditions were set like pre-infection probiotic treatment (aquarium tanks, temperature, filtered sea water, salinity, etc.). The only difference was that the probiotic treatment was carried out just after infection at week 3 (21 days).

Statistical analysis

The experimental data were subjected to statistical analysis using SPSS Statistics software package Ver. 25. The shrimp groups were analyzed using One Way ANOVA; and Post-hoc Tukey (LSD) analysis was used to compare the differences in the mean of these groups (at P-value of <0.05). All data were presented as means ± standard deviation (SD) of the duplicates.

Results

Morphological and agar well diffusion studies

In this study, the agar well diffusion method was used to assess the antimicrobial activity or microbial extracts. Based on results in Figure 2 (see panel A and B), gram staining showed that *L. lactis* appeared to be gram positive coccus while *V. parahaemolyticus* appeared as gram negative rod. The size of inhibition zone for *L. lactis* FA1 measured against *V. parahaemolyticus* in this study is approximately about ~5.0 mm (minus the well diameter of size ~5.2 mm) (Panel C). Results from morphological and inhibitory studies indicated that all these properties were consistent with previous observation made on these strains (Hamid *et al.,* 2020). This work also has verified the microbiological works that we used in this study, it is a gram-positive coccus, non-spore forming, and was reported to have probiotic properties (Bandyopadhyay *et al.,* 2022).

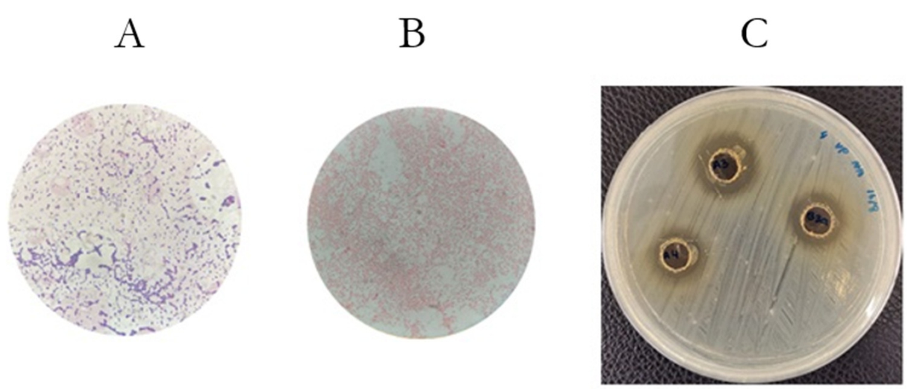


Figure 2. Gram staining of strains used (Panel A) *Vibrio parahaemolyticus* and (Panel B) *Lactococcus lactis*, viewed under light microscope (Olympus CH30) at 100× (Oil immersion). Panel C shows Agar well diffusion method with inhibition zones formed by *L. lactis* suspension against the lawn of *V. parahaemolyticus* grown on NA plates

Shrimp infection and survivability

For shrimp growth studies, the number of surviving shrimps were significantly affected by the type of treatment being given. In this study, *V. parahaemolyticus* strain has elicited AHPND symptoms towards shrimp *L. vannamei* as early as 3 days following infection. The infected shrimp frequently appeared symptom of pale to white hepatopancreas, and transparent body characteristic (see Figure 4 (A) and (B)). Some of the infected shrimp exhibited behavioural changes such as erratic swimming behavior. The erratic swimming behaviours were observed especially on the infected shrimps (Treatment B and C). As shown in Figure 3, the average number of surviving shrimps was 13 (~65%) for the control group which was given neither probiotics treatment nor being infected. There were also shrimps that were found dead following their jump out of the tank. Thus, it is unclear if all these shrimp deaths were due to infection. The infected shrimp were thus not necessarily end up in death as some shrimps were able to recover and loss the disease symptom. The number of surviving shrimps for Treatment A (Probiotic treated, non-infected) was 16 (~80%), higher than that recorded for Treatment B, at 13 (~65%) corresponding to probiotic treated but infected shrimp. The number of survived shrimps was the lowest for Treatment C (9, ~45%), corresponding to infected shrimp with no probiotic treatment. To those of recovered shrimps, they will appear healthy and their hepatopancreases regaining pigmentation, they have however failed to achieve the normal weight and length gains (see Figure 4).

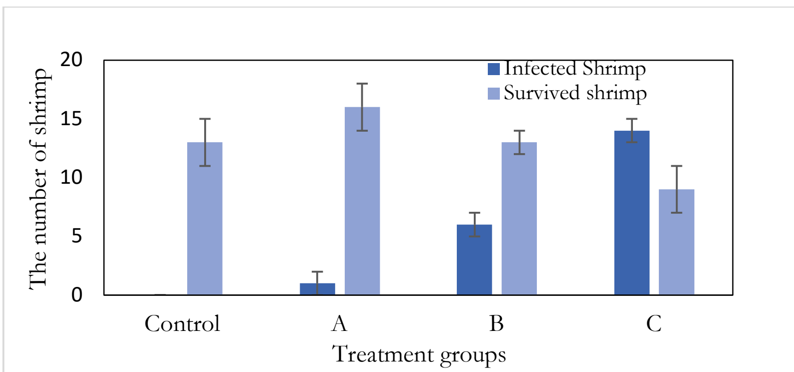
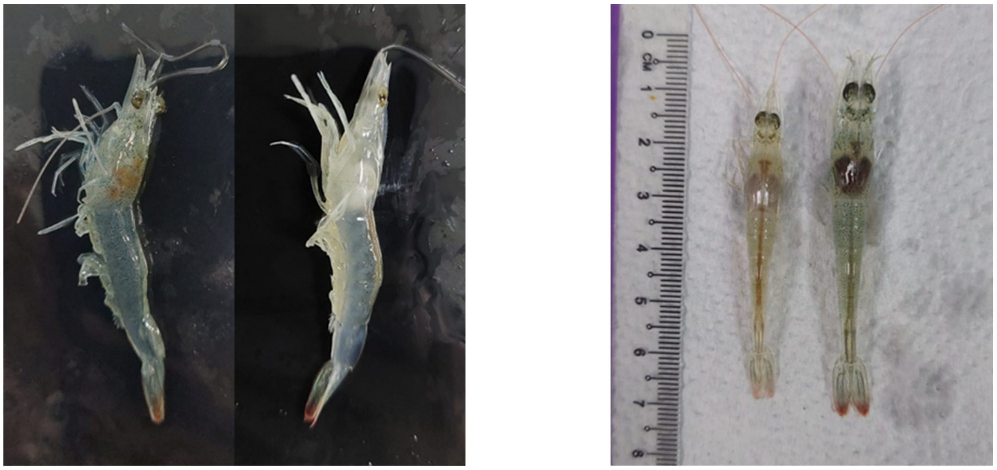


Figure 3. Infected and survived shrimp according to treatment group. The number of the infected and survived *L. vannamei* after being challenged with *V. parahaemolyticus*

The shrimp groups (Control, A, B and C) correspond to the treatments highlighted in Table 1. The figures represent the average shrimp number, and the error bars represent the standard deviation (SD) for each shrimp group.



A & B C

Figure 4. Comparison of hepatopancreas and the size of healthy and infected shrimp. The hepatopancreas of *L. vannamei* (marked with red circle) for a healthy (A); and infected (B) shrimp with AHPND symptoms. Panel (C) shows comparison of averaged sized for representative shrimp from Treatment C and Treatment A, pictures taken after 4 weeks of infection

Effect on feed conversion ratio, shrimp body weight and length gains

Table 3 shows the effect of probiotic supplement on shrimp feed conversion ratio (FCR), shrimp body weight and length gains. The FCR values for the shrimp groups fell within range of 1.5 to 2.8, in which group A showed the lowest value (1.53); meanwhile, group C had the highest value of 2.82. Generally, FCR for *L. vannamei* can range from 1.6 to 1.8 depending on the growth environments, and in a poor condition this value can increase up to 2.5 (Chaikaew *et al.,* 2019). Thus, group C (infected and untreated with probiotic) was considered the most inefficient in terms of nutrient consumption per weight gain. Figure 5 shows the effect of the probiotic on the shrimp growth measured by body weight and length gains. The weight gain of the shrimps that was continuously fed with probiotic (treatment A) for 4 weeks is the highest at 98.2% compared to the untreated control at 89.7%. The weight gain for shrimps under treatment B (continuously fed with probiotic but uninfected) was 94.41%, and this was higher than those of probiotic untreated but infected (treatment C) at 52.30%. As shown in statistical analysis using one way ANOVA on Table 3, there was significant difference in body weight gain but, less so in length gain. As shown in Table 4, Tukey post hoc (HSD) analysis showed that the difference between treatment A (probiotic treated, uninfected) and treatment C (infected and no probiotic) was significant, at 0.047 ($P < 0.05$). Meanwhile, a significant difference in weight gain was also observed between treatment B (infected and probiotic treated) and treatment C (infected and no probiotic) at 0.046 ($P < 0.05$). The effect of shrimp growth was also observable in terms of body length. The growth in body length showed similar trend seen in weight gain. Referring to Figure 5, probiotic treatment alone (treatment A) recorded the highest length gain, at 33.92%. This was secondly followed by infected shrimp supplied with probiotics at 27.2% (Treatment B) and the lowest was for infected shrimp without probiotic at 16.32% (treatment C). The control group recorded 25.7% length gain. Statistical evaluation on Table 4 showed no significant difference in the length gain between any pair of shrimp groups.

Table 2 The shrimp feed conversion ratio, their weight and length gains for each group

Treatment groups	Weight					Length			
	Initial weight (g)	Final weight (g)	Net weight gain (g)	Weight gain (%)	Feed Conversion Ratio (FCR)	Initial length (cm)	Final length (cm)	Net length gain (cm)	Length gain (%)
Control	0.88±0.06	1.68±0.24	0.796±0.14	89.8±18	1.89±0.25	5.07±0.06	6.37±0.55	1.30±0.30	25.7±23
A	0.99±0.09	1.97±0.12	0.978±0.10	98.2±11	1.53±0.20	5.13±0.12	6.87±0.20	1.73±0.16	33.8±9
B	0.98±0.11	1.93±0.15	0.936±0.13	94.9±14	1.60±0.23	5.27±0.36	6.70±0.26	1.43±0.31	27.2±21
C	1.00±0.05	1.54±0.03	0.532±0.04	52.9±80	2.82±0.10	5.16±0,27	6.13±0.15	0.96±0.21	18.7±10

*Each weight or length value is derived from the average weight or lenght values of each shrimp group. Each data is presented in average ±SD format.

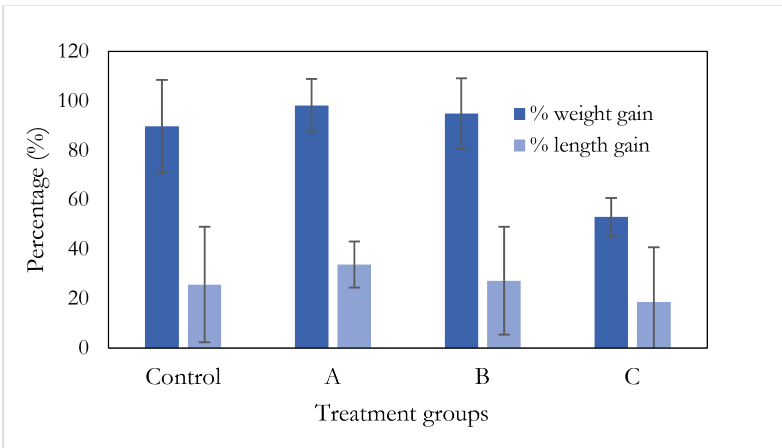


Figure 5. The percentage body weight and length gains of shrimps. The percentage of body weight and length gains for various treatment groups of *L. vannamei* were recorded after 4 weeks

The shrimp groups (Control, A, B and C) correspond to the treatments highlighted in Table 1. The figures represent the % values in shrimp number, and the error bars represent its standard deviation (SD).

Table 3. Analysis of shrimp length and weight gain of shrimp based using One Way ANOVA

One way ANOVA		Sum of Squares	df	Mean Square	F	Sig.
Body weight gain	Between Groups	0.408	3	0.136	4.741	0.035
	Within Groups	0.230	8	0.029		
	Total	0.638	11			
Changes in length	Between Groups	1.217	3	0.406	1.596	**0.265**
	Within Groups	2.033	8	0.254		
	Total	3.250	11			

*Bold figure indicates the significant less than P value of < 0.05.

Table 4. Post Hoc Tukey (HSD) analysis conducted on average body weight and length gains between all the treatment groups. At P value < 0.05, the average body weight gain for shrimp with Treatment A and B are significantly different from that of Treatment C (numbers indicated in bold). There is no significant difference in the length gain between treatment groups

Treatment	Treatment	Significant level (Weight gain)	Significant level (Length gain)
Control	A	0.563	0.725
	B	0.558	0.960
	C	0.298	0.725
A	Control	0.563	0.725
	B	1.000	0.939
	C	**0.047**	0.230
B	Control	0.558	0.960
	A	1.000	0.939
	C	**0.046**	0.460
C	Control	0.298	0.725
	A	**0.047**	0.230
	B	**0.046**	0.460

Post-infection probiotics administration

Another similar study was also conducted, whereby probiotic treatment was given only after the shrimp infection. The results (Supplementary) summarize the outcome from this study. The number of surviving shrimps was maximum for the control group, compared to the other 3 treatment groups (A, B and C). Like in pre-infection study, treatment C (probiotic untreated, infected) recorded the lowest number of surviving shrimps, and the highest number of infected shrimps. Finally, the statistical analysis to compare different groups was evaluated and shown in Table 5.

Table 5. Analysis of shrimp length and weight gains for post-infection probiotic treatment of shrimp using One Way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Weight gain	Between Groups	0.373	3	0.124	0.900	0.515
	Within Groups	0.552	4	0.138		
	Total	0.925	7			
Length gain	Between Groups	0.933	3	0.311	0.580	0.658
	Within Groups	2.143	4	0.536		
	Total	3.075	7			

Discussion

AHPND symptoms in shrimp

Infected shrimp are characterized by exhibition of key symptoms such as lethargy, anorexia, slow growth, empty digestive tract, a pale to white hepatopancreas, disappearance of hepatopancreas and shrimp death (Anjana and Tiwari, 2022). The shrimp was considered infected if they appeared to have any of these symptoms. Due to the presence of symptom that was not necessarily resulted in shrimp death, we speculated that the infection elicited by this *V. parahaemolyticus* strain was rather mild. We also observed that symptomatic shrimp reverting to healthy shrimp with disappearance of symptoms after two weeks of infection, especially to those of probiotic treated group (treatment B). The erratic swimming behaviour observed especially in treatment group B and C were associated with lethargy, commonly observed on shrimp infected with EMS (Zorriehzahra and Banaederakhshan, 2015).

Shrimp survivability studies

The use of *L. lactis* strain is consistent with many studies using other probiotic strains which generally resulted in improved the survivability, feed conversion and specific growth rate of Penaeid shrimp (Toledo *et al.,* 2019). By comparing treatment A with control group, probiotic supplement does bring benefit even to the healthy and uninfected shrimp. In this study, the mortality within the 4 weeks period for treatment C (infected shrimp with no probiotic) was however not as high as 100%. This could be due to the lesser virulence of *V. parahaemolyticus*. Although this strain was originally collected from AHPND infected shrimp, the virulence could have been lost over time. Less virulent strain has caused a milder symptom that normally resulted in less than 100% mortality in shrimp (Soto-Rodriguez *et al.,* 2015). Thus, with a milder infection, probiotic supplementation has not only resulted in reduction in mortality, but it also helped in the recovery of the infected shrimp. This explained the reversion of infected shrimp into healthy shrimp we observed in treatment B.

Effect of probiotics on shrimp body weight and length gains

The study on weight gain indicated that probiotic supplement gave a positive effect on the shrimp growth by increasing the shrimp weight gain. Nevertheless, the highest weight gain was achieved in probiotic only treated shrimp (treatment A) implying that probiotic could provide it maximal effect on healthy shrimp compared to infected shrimp. In several studies using other bacterial species, probiotics has been shown to bring a lot of benefit to the growth of *L. vannamei* (Amiin *et al.,* 2023). One reason is that probiotics could aid in food digestion via breaking down complex into simpler molecules such that it can be easily absorbed in the intestine. Probiotics improve growth performance which is observable through weight gain, length gain, and feed conversion ratio. For instance, treatment of probiotic to shrimp increase the growth performance especially in the dry weight gain (Wang and Gu 2010). The changes in shrimp body lengths were however smaller than changes in their body weight. Even though the changes in body length have similar trend compared to the changes in body weight gain, changes in these two parameters were not directly proportional. The shrimp has acquired more weight gain compared to length gain per unit time. This can be referred to as positive allometry growth pattern due to the fact that the shrimp had acquired more weight gain compared to length (Salim *et al.,* 2020). The statistical evaluation on the changes in either body length or weight could be improved by using larger shrimp population size.

Pre-infection versus post-infection administration of probiotics

With post-infection administration of probiotics, for treatment A (probiotic treated, uninfected), there was not much improvement in either weight or length gain when compared to that of control group. This was in contrast to observation made in pre-infection administration of probiotics. Thus, this method is rather ineffective compared to pre-infection exposure of probiotics. There were also similar trends in both weight and length gains. Comparing treatment C (No probiotics, infected) with other groups, despite there were still decreases in both weight and length gains, these changes were however statistically insignificant, as shown by P-values greater than 0.05. Again, this study was limited due to the small sample size that we have used, which can be improved by increasing the shrimp population numbers.

Inhibitory mechanism of L. lactis strain towards V. parahaemolyticus

The supplementation of *L. lactis* strains on shrimp can affect shrimp growth in two ways, firstly by enhancing the shrimp growth and secondly, by alleviating disease symptoms on infected shrimp. Despite of these observations, it is uncear on how actually the suppression of pathogen is accomplised. Probiotic strains were known to benefit host by several mechanisms such as competitive exclusion, surface hydrophobicity, bile acid tolerance, and stimulation of shrimp's immune response (Ganguly *et al.,* 2018). Our previous work has demonstrated that this strain produces bacteriocin and it is able to antagonise *V. parahaemolyticus* on petri dish (Hamid *et al.,* 2020). Whether the bacteriocin is the key factor enabling these inhibitory properties in shrimp will remain to be elucidated. Ability of a probiotic strain to withstand acid and bile was a pre-requisite requirement for a probiotic strain, especially on vertebrates (Ganguly *et al.,* 2019). This is also true considering this strain was originally isolated from shark, and this strain was shown to be bile tolerant (Bandyopadhyay *et al.,* 2022). Even though shrimp does not produce bile, it may be added as supplement that can benefit its nutrient intake. Thus, future use of this strain in the presence of bile on invertebrate like shrimp should be further evaluated.

Conclusions

Probiotic supplementation with *L. lactis* strain on white-legged shrimp *Litopenaeus vannamei* was able to improve the survivability of shrimp upon being challenged with pathogen *V. parahaemolyticus* strain. The infected shrimp exhibiting a milder form of AHPND/EMS symptoms were able to show recovery upon probiotics treatment. Shrimps treated with probiotics have shown significant weight gain compared to infected shrimp without probiotic treatment. Probiotics supplement could also render its effects on non-infected or healthy shrimp. This study also indicated that an earlier exposure to probiotics can be more effective in disease prevention, compared to the treatment only after infection. This indicated that the *L. lactis* strain is highly suitable in developing probiotics strain that can function as biocontrol agent in managing EMS diseases for shrimp aquaculture. Moreover, this strain offers several advantages compared to *Bacillus* sp. strain, commonly used in managing shrimp diseases.

Authors' Contributions

The contributions of authors to the manuscript should be specified in this section; according to the type of contribution (choosing only the appropriate ones), the authors are mentioned by initials: Conceptualization: THTAH and MFN; Data curation NASB, WAWAG and FCO; Formal analysis; THTAH; Funding acquisition: THTAH and MFN; Investigation THTAH; Methodology; MFN and FCO; Project administration: THTAH and MFN; Resources: THTAH and MFN; Software: FCO; Supervision: THTAH, FCO and MFN; Validation: THTAH and FCO; Visualization: NASB and WAWAG; Writing - original draft THTAH; Writing - review and editing THTAH and FCO.
All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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