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Original Article

Dietary supplementation of *Lactobacillus* sp. and garlic extract on growth performance, digestive enzyme, and disease resistance of the whiteleg shrimp, *Litopenaeus vannamei*

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Abstract: In the present study, the effects of a probiotic (*Lactobacillus* sp.), a prebiotic (1% garlic extract), and their combination were evaluated in whiteleg shrimp (*Litopenaeus vannamei*) using a feeding supplement. The shrimp in each tank were fed the following experimental diets for 56 days: the control diet (commercial feed) and experimental diets supplemented with *Lactobacillus* sp. (diet 1), garlic extract (diet 2), and *Lactobacillus* sp. + garlic extract (diet 3). The results showed that shrimp fed diets 1, 2, and 3 had higher growth indices, including weight gain, specific growth rate, and daily weight gain. The supplemented diet 3 recorded the lowest FCR and the highest shrimp survival rate after 56 days of feeding. In addition, the activities of amylase, protease, and aminopeptidase were significantly increased in shrimp fed the experimental diet relative to the control diet from day 28 until the end of the experiment. The overall bacterial count of *Vibrio* was significantly lower in diets 1, 2, and 3 than in the control diet at day 56. Moreover, shrimp fed diet 3 had the lowest cumulative mortality after 96 h post-challenge with *Vibrio parahaemolyticus*. Overall, the findings demonstrated that the addition of synbiotics containing 10^8 CFU/kg *Lactobacillus* sp. and 1% garlic extract could promote growth, survival, and disease resistance in whiteleg shrimp reared under tank conditions.

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Introduction

The shrimp industry has become one of the most economically important sectors of aquaculture in many countries worldwide. The global production of penaeid shrimps via aquaculture has increased from 6056 thousand tons in 2018 to 7934 thousand tons in 2022 (FAO, 2024), in which whiteleg shrimp, *Litopenaeus vannamei* was the top species produced, with 6.8 million tonnes in 2022. In Vietnam, production has increased rapidly in recent years, with the successful adoption of semi-intensive and intensive systems. However, these high-density stocking systems not only adversely affect the health of farmed shrimp but also exacerbate the risk of disease. Dietary supplementation with medicinal plants, probiotics, and prebiotics has been evaluated in aquaculture as an effective method to replace antibiotics, thereby stimulating growth and preventing disease (Hill et al., 2014).

Garlic (*Allium sativum*) is a well-known medicinal plant widely cultivated worldwide for its medicinal and nutritional properties. It is rich in bioactive substances, including polysaccharides, sulfur-containing organic compounds, amino acids, and polyphenolic compounds (Zhang et al., 2024), which are associated with antioxidant, antimicrobial, antifungal, and anti-inflammatory properties as well as immunomodulatory effects in aquatic animals (Gabriel et al., 2021; Rezaei et al., 2022; Güroy et al., 2024). Previous studies have reported positive effects of garlic on growth performance, digestive enzyme activity, immune responses, and intestinal microbial flora in fish and crustacean species (Lee and Gao, 2012; Safari and Paolucci, 2017; Tazikeh et al., 2020). Supplementation of garlic water extract at 1% in a whiteleg shrimp diet (Phan et al., 2023) and at 2% (20 g/kg) in a freshwater prawn (*Macrobrachium rosenbergii*) diet (Jahanbakhshi et al., 2024) improved

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growth performance and feed conversion ratio. In addition, polysaccharides from garlic bulb extract may regulate gut microbial activity (Lu et al., 2021) and serve as prebiotics to stimulate the growth of beneficial bacteria (Sunu et al., 2019). Supplementation of *Fenneropenaeus indicus* diets with 1% garlic extract increased resistance to experimental infection with *Vibrio harveyi* (Vaseeharan et al., 2011). Elbaz et al. (2021) reported that garlic could reduce pathogenic bacteria, such as *Escherichia coli* and coliforms, and increase Lactobacilli in the gut of broiler chickens.

Probiotic *Lactobacillus* species are commonly used as feed supplements to promote growth and control diseases in shrimp and fish aquaculture (Wei et al., 2022). Kongnum and Hongpattarakere (2012) reported that the addition of *L. plantarum* MRO3.12 isolated from the intestine of wild banana shrimp enhanced the growth performance, feed efficiency, and survival of whiteleg shrimp after exposure to *V. harveyi*. In addition, dietary administration of *L. plantarum* could modulate the intestinal microbiota in *L. vannamei,* showing an increased abundance of beneficial bacteria (Zheng et al., 2020). In recent years, medicinal plants and probiotic mixtures have been used in aquaculture for their ability to enhance growth, immunity, and pathogen resistance in aquatic species (Wei et al., 2022). Yu et al. (2009) reported that whiteleg shrimp exhibit higher specific growth rates after dietary supplementation of *Bacillus* and medicinal herbs compared with bacillus or herbs alone. Furthermore, the addition of synbiotics from garlic extract and *L. acidophilus* increased weight gain, immune function, intestinal health, and antioxidant levels in broiler chickens (Sunu et al., 2021). However, little is known about the effects of dietary supplementation with a combination of garlic extract and *Lactobacillus* sp. on whiteleg shrimp. Therefore, this study evaluated the effects of garlic water extract and *Lactobacillus* sp., individually and in combination, on growth parameters, digestive enzyme activity, intestinal microbiota counts, and disease resistance of whiteleg shrimp against *V. parahaemolyticus*.

Materials and Methods

Preparation of the experimental diets: Garlic bulbs were extracted as described by Ng and See (2019), with modifications. In summary, dried garlic was finely powdered and extracted with hot water at 50^{o}C for 20 min. After centrifugation, the extract was freeze-dried by a Freeze Dryer (BenchTop Pro 9L EG, UK) and stored at -4^{o}C until using experimental diets.

Lactobacillus sp. isolated from the intestinal tract of whiteleg shrimp in a semi-extensive system in Ca Mau, Vietnam, was used in the present study. *Lactobacillus* sp. was stored at -80^{o}C at the laboratory of probiotics, College of Aquaculture and Fisheries, Can Tho University. The bacterium was cultured in MRS broth medium and then centrifuged to collect the bacterial suspension. Afterwards, the pellets were homogenized and adjusted to 1×10^9 CFU/mL (OD_{nm}=1.5) using a spectrophotometer (Shimazu, UV-1900i, Malaysia). Four different experimental diets were prepared, including a control diet (commercial feed), diet 1 (control diet supplemented with 1% garlic extract), diet 2 (control diet supplemented with *Lactobacillus* sp. 10^8 CFU/kg), and diet 3 (control diet supplemented with 1% garlic extract and *Lactobacillus* sp. 10^8 CFU/kg). The supplemented diets were prepared as described previously (Phan et al., 2024). Briefly, the required amounts of garlic extract and/or *Lactobacillus* sp. were gently sprayed on commercial feed and air-dried at room temperature before storing in a freezer at -4^{o}C for feeding the shrimps.

Experimental design: Whiteleg shrimp post-larvae were collected from the wetlab at the College of Aquaculture and Fisheries. They were placed in a 4 m^3 composite tank with aerated brackish water (salinity of 15 ppt, pH = 8.0-8.2, and temperature of 28.2-29.0^{o}C and fed a commercial shrimp diet (Growmax Vietnam, Co., Ltd., 40% protein, 5% lipid, and 11% moisture). The experiment was conducted in an indoor wet lab under natural daylight, and four treatments were replicated three times.

Juvenile shrimp were transferred to a 500-L composite tank with a density of 100 individuals. Experimental shrimp were fed different treatment diets for 56 days. Shrimp were fed the respective diets at 3-7% of their wet body weight. During the trial, water quality, including temperature, pH, DO, and alkalinity, was maintained at 28.3-29.2°C, 7.9-8.2, and 4.5-5.1 mg/L, and 126.8-132.1 mg$CaCO_3$/L, respectively. TAN (total ammonium nitrogen) concentration fluctuated between 0.304 and 0.80 mg/L, NO_2^--N (nitrite) at 0.031 to 0.445 mg/L, and NO_3^--N(nitrate) at 0.314 to 3.53 mg/L, which were in the acceptable range for shrimp species (Lin and Chen, 2003).

Data collection

Growth and survival parameters: The weight of shrimp in all treatments was measured at the start and termination of the experiment to calculate the growth parameters as follows (Tu et al., 2023):

Weight gain (g) = final weight - initial weight

Specific growth rate (SGR, %/day) = ((LnFinal weight - LnInitial weight)/day of culture) x100

Daily growth rate (DWG, g/day) = (Final weight - Initial weight)/day of culture)

Final biomass (kg/m^3) = number of shrimp x final weight

Survival (%) = (total number of initial shrimp/total number of final shrimp)/100

Feed conversion ratio (FCR) = feed intake /weight gain

Digestive enzyme activity: Intestinal shrimp was weighed and placed in an Eppendorf tube containing sterile solution (0.85%) at a ratio of 1:9 (w/v) and then centrifuged at 2500×g at 4°C for 10 min to harvest the supernatant for analyzing enzyme activity. The total amount of protein in the intestinal samples was determined based on Bradford (1976) method using BSA as a standard. Enzyme activities were analyzed based on the method of Lowry et al. (1951) for protease, Bernfeld (1955) for amylase, and Ezquerra et al. (1999) for leu-aminopeptidase activity. Protease, amylase, and leu-aminopeptidase activities were measured using a microplate reader (USA) by monitoring changes in absorbance at 660 nm, 540 nm, and 410 nm, respectively, and were expressed as specific activity (U/mg protein).

Intestinal bacteria density: Total bacteria, *Vibrio* spp., and *Lactobacillus* spp. counts in the shrimp's intestine were analyzed using a plate counting method following the standard method of APHA (2017). In summary, 100 µL of a homogenized intestine solution was gently spread on the TSA (Tryptone Soya Agar) plate for total bacteria, MRS plate for *Lactobacillus* spp., and TCBS plate for *Vibrio* spp. counts. Nine shrimp from each treatment were randomly collected for analysis. In total, 108 shrimp were used in this study.

Challenge test: A pathogenic bacterium, *V. parahaemolyticus*, was prepared as in our previous study (Phan et al., 2024). In brief, the bacteria were cultured in tryptic soy broth (TSB), then centrifuged and adjusted to 1×10^7 CFU/mL. 30 shrimp from each tank (three tanks for each diet group) were used for this trial, and 20 µL of this bacterial suspension was injected into the abdominal segment of the shrimp. Shrimp were transferred to a plastic tank containing 100 L of seawater at 15 ppt after injection. As a negative control, 30 shrimp were injected with a sterile solution (0.85%) at the same volume as the bacteria. Dead shrimp were observed and removed every 6 hours until 96 h post-challenge. The cumulative mortality of shrimp was calculated at 12, 24, 48, 72, and 96 hours.

Statistical analysis: Data were checked for normal distribution with Shapiro-Wilk's test, and homogeneity of variances was verified with Levene's test. Results were compared using a one-way ANOVA followed by a post-hoc test (Tukey's test) for multiple comparisons using SPSS software (Version 22). Statistically significant differences were considered when $P<0.05$.

Results

Growth performance: Table 1 shows the growth parameters and survival of whiteleg shrimp fed dietary *Lactobacillus* sp. and/or garlic extract for 56 days. Final weight, WG, DWG, and SGR of shrimp were significantly higher in the diets 1, 2, and 3 than the control diet ($P=0.016$, df=3 for FW; $P=0.016$, df=3 for WG; $P=0.015$, df=3 for DWG; $P=0.040$, df=3 for SGR). However, no significant differences were

Table 1. Growth performance and survival of the whiteleg shrimp after 56 days of feeding experimental diets.

Parameters	Treatments			
	Control	Diet 1	Diet 2	Diet 3
Initial weight (g)	0.440±0.002[a]	0.440±0.002[a]	0.440±0.008[a]	0.440±0.005[a]
Final weight (g)	14.43±0.51[a]	15.81±0.26[b]	15.86±0.12[b]	15.93±0.05[b]
WG (g)	13.99±0.51[a]	15.36±0.26[b]	15.41±0.11[b]	15.48±0.06[b]
DWG (g/day)	0.250±0.009[a]	0.274±0.011[b]	0.275±0.015[b]	0.276±0.005[b]
SGR (%/day)	6.217±0.072[a]	6.385±0.034[b]	6.389±0.017[b]	6.396±0.025[b]
Survival rate (%)	73.7±0.9[a]	79.3±0.8[b]	79.0 ±1.00[b]	84.7±0.8[c]
Biomass (kg/m³)	2.51±0.12[a]	3.00±0.06[b]	2.97±0.04[b]	3.17±0.03[b]
FCR	1.45±0.06[a]	1.22±0.02[b]	1.21±0.01[b]	1.04±0.02[c]

Data are expressed as mean ± SD. Different letters within the row indicate significant differences among treatments (P<0.05).

observed in these parameters among diets 1, 2, and 3. A similar result was observed in the biomass of shrimp, whereas the values in all experimental diets were greater than in the control diet. The highest survival rate was observed in diet 3, which differed significantly from the others. Moreover, a significant decrease in FCR was recorded in diets 1, 2, and 3 compared to the control diet (P=0.0001, df=3).

Digestive enzyme activity: The digestive enzyme activity of the intestinal samples throughout the experiment is shown in Figure 1. The f amylase in the experimental diets showed an increase from the beginning (day 1) to the middle of the feeding period (day 28) and then slightly reduced to the end of the study (day 56) (Fig. 1A). The activity of amylase in diets 2 and 3 reached the highest values on day 28. It was significantly different than the diet 1 and the control diet (P=0.0003, df=3). On the 14th day of the feeding period, protease activity in the experimental diets was noticeably higher than in the control diet. There were no significant differences in protease activity among experimental diets at day 28 and day 56 (Fig. 1B). As for leu-aminopeptidase activity, it showed an increase in all treatments from day 1 to day 56 of the culture period. At day 28, the level of leu-aminopeptidase in the experimental diets was significantly higher compared to the control diet (p=0.001, df=3), but no significant effect was recorded among experimental diets (diets 1, 2, and 3). The same result was observed at the termination of the experiments. In addition, the greatest value of leu-aminopeptidase activity was recorded in diet 3 at day 42 (Fig. 1C).

Intestinal bacteria population: The density of *Vibrio* spp. in the intestinal sample was significantly lower in the experimental diets compared to the control diet (P=0.0004, df=3). However, an insignificant difference was detected between the experimental diets at the end of the feeding period (Fig. 2a). There was a significant increase in *Lactobacillus* spp. Density in the diets 1, 2, and three groups relative to the control diet at day 56 (P=0.0001, df=3). The highest level of *Lactobacillus* spp. was observed in the diet 3 at the end of study (Fig. 2b). The similar pattern was observed in total bacteria count, in which the values in diets 2 and 3 were significantly greater than in the control and diet 1, but there was no significant difference between these groups (P=0.0005, df=3) (Fig. 2c).

Mortality of shrimp after *V. parahaemolyticus* injection: No mortality was observed in the unchallenged test groups (control negative) throughout the trial. The mortality rate started to increase at 24 h of injection, and significant differences were observed among treatments (Fig. 3). At 96 h, the cumulative mortality rate of the shrimp fed diet 3 injected with *V. parahaemolyticus* was 30.0%, which was significantly lower than that of the control diet, diet 1, and diet 2 (P=0.0000, df=3). However, no significant difference in mortality rates was observed between shrimp fed diets 1 and 2.

Discussions

Numerous studies have shown that probiotics from Lactobacillus species have beneficial effects on shrimp when administered at adequate doses in the diet (Zheng et al., 2018; Phan et al., 2024). It has also been shown that garlic extract can effectively enhance

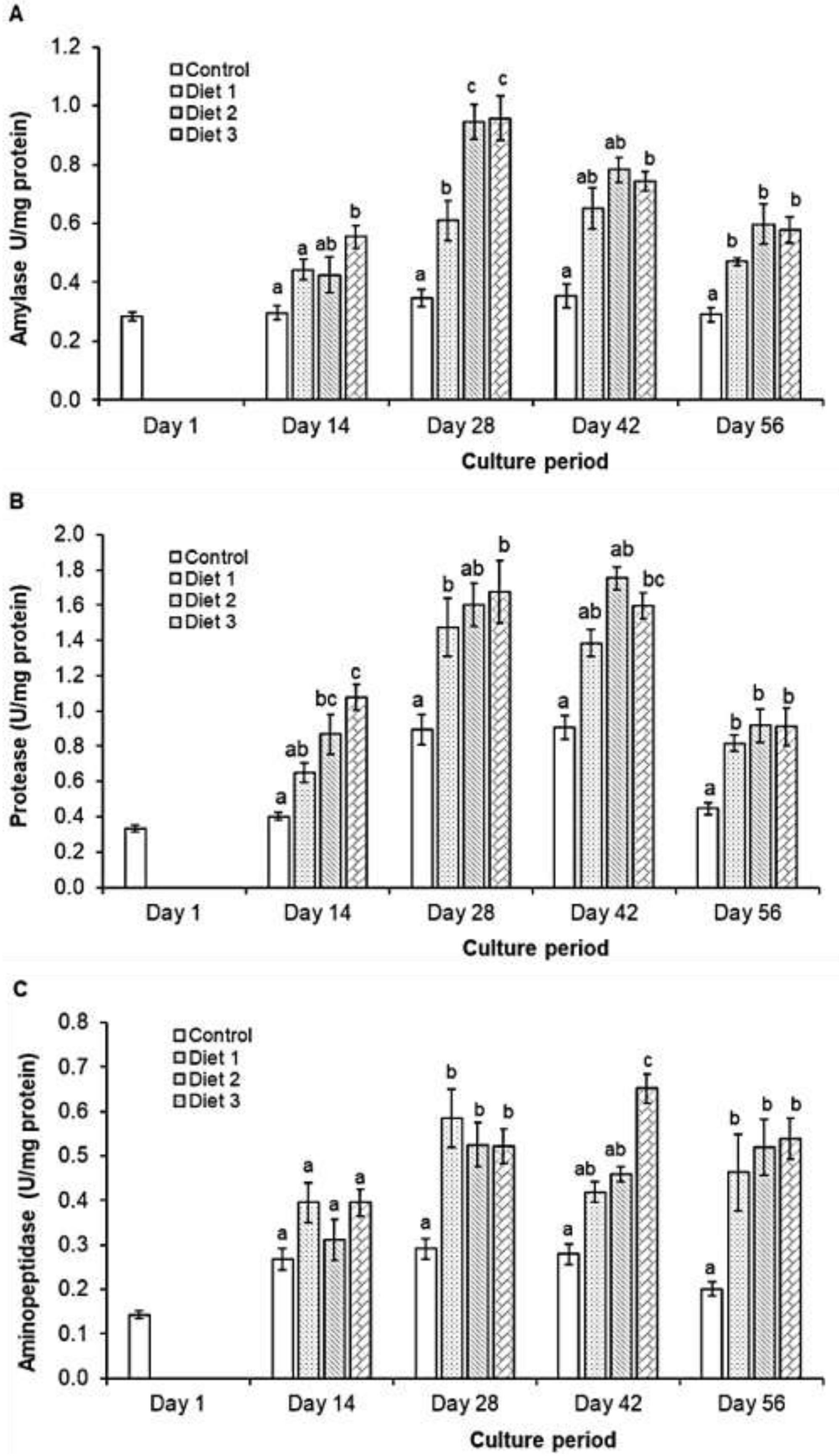

Figure 1. Amylase, protease, and aminopeptidase activity of whiteleg shrimp during the culture period. Data are expressed as mean±SD (n=9). Different letters above the bar indicate significant differences among treatments in the same period (*P*<0.05).

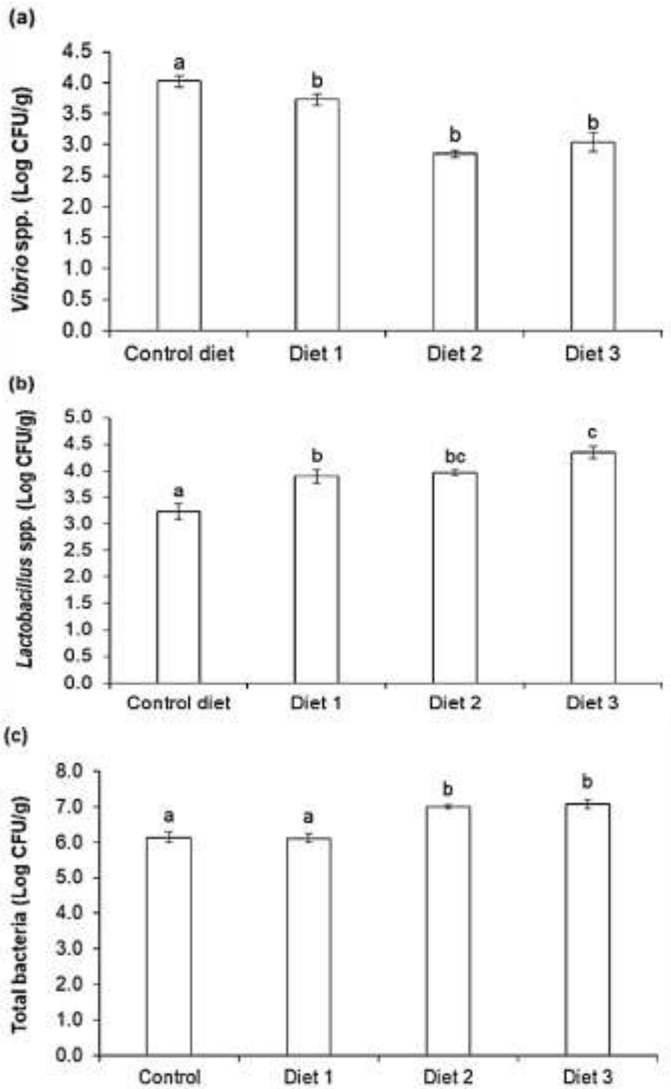

Figure 2. *Vibrio* spp. (a), *Lactobacillus* spp. (b), and total bacteria (c) counts in the intestine of whiteleg shrimp after 56 days of feeding experimental diets. Data are expressed as mean±SD (n=9). Different letters above the bars indicate significant differences among treatments (P<0.05).

shrimp growth performance and digestive enzyme activity (Pazir et al., 2018; Chirawithayaboon et al., 2020; Phan et al., 2023). Chin et al. (2024) found that the combined supplementation of 10^8 CFU/mL *L. plantarum* and 2% seaweed *Sargassum polycystum* in diets showed a positive impact on *P. monodon* immunological responses and resistance against *V. parahaemolyticus*. Similarly, the addition of chitosan and complex probiotics (*L. plantarum, B. amyloliquefaciens, B. pumilus*, and *B. subtilis*) significantly enhanced immune response-related gene expression, including ALF, proPO, SOD, and LYZ, and disease resistance to *V. parahaemolyticus* (Kewcharoen and Srisapoome, 2022). In the present study, we investigated the effects of synbiotics derived from *Lactobacillus* sp. and garlic extract on growth parameters, survival, digestive enzyme activity, and resistance to pathogenic bacteria of whiteleg shrimp. The findings suggested that supplementation with Lactobacillus sp. and/or garlic extract (diets 1, 2, and 3) significantly enhanced final body weight, weight gain, daily weight gain, and specific growth rate in whiteleg shrimp. Incorporating 1% garlic extract and *Lactobacillus* sp. at 108 CFU/kg into the diet resulted in a higher survival rate and lower FCR than diets supplemented with garlic extract or *Lactobacillus* sp. alone after 56 days of culture. This finding is consistent with Chin et al. (2024), who reported that a combination of probiotic L. plantarum and prebiotic S. polycystum in the diet improved FCR and survival in black tiger shrimp, *P. monodon*. The improved FCR in shrimp fed a synbiotic containing *Lactobacillus* sp. and garlic extract indicated better nutrient digestibility, which may be due to increased intestinal villus height. Boonanuntanasarn et al. (2016) reported that the combined addition of β-glucan with *B. subtilis* or *Pediococcus acidilactici* in diets resulted in significantly higher villi height than the individual addition. A decrease in FCR is expected to result in lower feed supplementation, thereby potentially reducing production costs and reducing environmental waste. De Silva and Hasan (2007) reported that feed accounts for 40-60 percent of total production costs in aquaculture systems.

In the present study, dietary co-supplementation with *Lactobacillus* sp. and garlic extract significantly increased the level of *Lactobacillus* spp. and total bacterial counts in the shrimp's intestine. Similarly, Prabawati et al. (2022) reported that the combination of supplementation with probiotic *L. plantarum* and the herb king oyster mushroom, *Pleurotus eryngii,* in the diet greatly improved the presence of *Lactobacillus* bacteria in the gut of whiteleg shrimp compared with those fed a diet supplemented with *P. eryngii* or *L. plantarum* alone. In addition, Nurhayati et al. (2015) demonstrated that the dietary supplementation of synbiotics derived from sweet

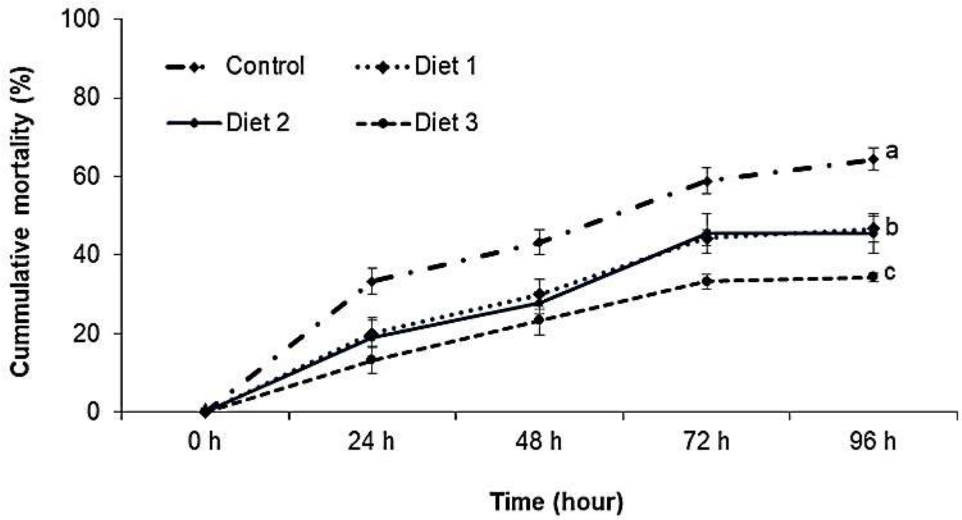

Figure 2. Cumulative mortality of the whiteleg shrimp fed with different experimental diets for 56 days following *Vibrio parahaemolyticus* challenge. Data are expressed as mean±SD (n=3). Different letters in the same period indicate significant differences among treatments (*P*<0.05).

potato (*Ipomoea batatas L.*) and probiotics significantly improved the total bacterial count in the digestive tract of whiteleg shrimp. In contrast, shrimp fed *L. plantarum*, garlic extract, and their combination produced a significantly lower *Vibrio* count in the gut than other shrimp fed the control diet. The finding is similar to that of Munaeni et al. (2014), who reported that the total number of Vibrio bacteria in the treatment supplemented with synbiotics containing Bacillus NP5 RfR and sweet potato was lower than in the control. Furthermore, Sarida and Harpeni (2010) reported that a decrease in *Vibrio* counts in the intestine is associated with increased survival rates in whiteleg shrimp.

The supplementation of *Lactobacillus* sp. and garlic tends to increase the activity of amylase, protease, and aminopeptidase in whiteleg shrimp following shrimp growth. In addition, the administration of *Lactobacillus* sp. and garlic extract more effectively promoted digestive enzymes in whiteleg shrimp than their individual administration, suggesting a synergistic effect between the beneficial bacteria and the prebiotic, garlic extract. A possible explanation is that garlic exhibits a prebiotic effect that stimulates the growth of beneficial bacteria, thereby enhancing digestive enzyme activity. In fact, garlic is rich in bioactive compounds such as allicin, allin, S-allyl-cysteine, saponin, flavonoid, and others that promote the growth of the *Lactobacillus* genus (Sunu et al., 2019; Kubba et al., 2021). Moreover, Chattaraj et al. (2022) indicated that *Lactobacillus* species can secrete digestive enzymes and modulate the intestinal microbiota in aquatic species. Several studies have also reported that elevated digestive enzyme activity in response to dietary synbiotic supplementation in other shrimp species, including *P. monodon* (Chin et al., 2024) and *M. rosenbergii* (Li et al., 2024). In the present study, enhanced amylase, protease, and aminopeptidase activities, supplemented by *Lactobacillus* sp. and garlic extract in the diet, resulted in improved feed digestibility of carbohydrate, protein, and peptide substrates.

The cumulative mortality rate from *V. parahaemolyticus* was lower in shrimp fed a diet containing garlic extract and *Lactobacillus* sp., individually or in combination; however, the lowest mortality rate was recorded in shrimp fed diet 3, which contained garlic extract with *Lactobacillus* sp. A study by Siddik et al. (2022) reported that adding garlic and probiotics of the genus *Lactobacillus* to the diet reduced mortality in juvenile barramundi, Lates calcarifer, following a *V. harveyi* challenge. Similarly, several studies have demonstrated that the administration of synbiotics significantly improved disease resistance to pathogenic bacteria in other shrimp species, including kuruma shrimp *P. japonicus*

(Zhang et al., 2011), giant freshwater prawn, *M. rosenbergii* (Muthukrishnan et al., 2020), and black tiger shrimp, *Penaeus monodon* (Chin et al., 2024).

The findings also indicated that shrimp fed synbiotics were better protected against *V. parahaemolyticus* than those fed garlic or *L. plantarum* alone, suggesting a synergistic effect between garlic and L. plantarum. As mentioned above, garlic contains a variety of nutrients that stimulate the growth of the *Lactobacillus* sp. and reduce pathogenic bacteria in the host by competing with their adherence and colonization (Sunu et al., 2019; Kubba et al., 2021), promoting the host's protection against diseases (Preidis et al., 2011). Furthermore, the bioactive compounds in garlic, including allicin and alliin, are implicated in protecting against pathogenic bacteria, which could improve the health of aquatic animal aquaculture species (Valenzuela-Gutiérrez et al., 2021).

In conclusion, dietary *Lactobacillus* sp. and garlic extract administration, individually or combined, increased growth performance, digestive enzyme activities, and beneficial bacteria in the intestine of whiteleg shrimp and decreased FCR after 56 days of feeding. In addition, the supplementation with *Lactobacillus* sp. combined with or without garlic extract markedly reduced the mortality of whiteleg shrimp after injection with pathogenic *V. parahaemolyticus*. Importantly, dietary administration of 1% garlic extract and 108 CFU/kg Lactobacillus sp. is an optimal dose, resulting in the greatest enhancement in survival rate, FCR, and resistance to pathogenic bacteria.

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