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Well-defined multispecies probiotic and enzyme combination outperforms traditional fermented probiotic applications in an intensive Pacific white shrimp, *Litopenaeus vannamei* (Boone, 1931), culture system

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Abstract

A series of treatments were designed to evaluate the efficacy of feeding commercial multispecies probiotics feeding with enzymes and fermentation process on the growth parameters and culture environment of Pacific white shrimp, *Litopenaeus vannamei* (Boone, 1931), in an intensive culture system. Commercial multispecies probiotics and enzymes (PEs) were continuously applied in three different doses, namely (i) 0.2, (ii) 0.4, and (iii) 0.6 mg L^{-1} and designated as 0.2, 0.4, and 0.6 PE during the first 30 days of intensive culture of Pacific white shrimp, *L. vannamei* (Boone, 1931). The probiotics were continually applied every alternate day, while the enzymes were added every sixth day throughout the trial period. The PE dose for all treated tanks was increased by 0.2 ppm after 30 days of culture and another 0.2 ppm after day 60. Meanwhile, fermentation technique, which has become the common method applied in Indonesia in the control treatment, was added on the same day with the PE group with increasing dosage, following the same trend with the 0.6 PE group. Results showed that the group receiving 0.6 PE showed

higher final biomass, higher mean weight, and protein retention efficiency as well as a lower feed conversion ratio compared with the control treatment. Abundance of *Vibrio* spp. remained below 10^3 cfu mL^{-1} throughout the trial. Water quality indicators TAN, NO_2—N and NO_3—N peaked in weeks 3–5 and then declined after that until the end of the culture period in all tanks. This decline was significantly faster in PE-treated tanks. Multispecies PEs have potential applications in controlling *Vibrio* spp., maintaining proper water quality condition, and enhancing the growth of shrimp in intensive culture system.

KEYWORDS
bioremediation, enzyme, growth, *Litopenaeus vannamei*, probiotics

1 | INTRODUCTION

Recently, the production of Pacific white shrimp, *Litopenaeus vannamei* (Boone, 1931) has shifted from extensive to intensive production systems, characterized by the use of high stocking densities of post-larvae (PL) ranging from 110 to 500 PL m^2 for intensive, and >500 PL m^2 for supra-intensive farming system (Zulkarnain et al., 2020). The technology used in the production system can increase the yield from <1 ton ha^{-1} in extensive system to 10–42 ton ha^{-1} in an intensive farming system (Lailiyah et al., 2018; Wahyudi et al., 2019). However, with increasing stocking density, the risk of disease transmission also heightens along with the poorer water quality during the culture period (Hill, 2002; Samocha, 2019). These stressful conditions can lead to substantial economic losses due to the high mortality of aquatic organisms (Bondad-Reantaso et al., 2005) and the use of therapeutic medicines to overcome the stressful production conditions (Wang et al., 2008). Considering the overuse of antibiotics and subsequent risks of antimicrobial resistance and residues (Aldeman & Hastings, 1998; Cabello, 2006), novel prophylactic approaches are needed to replace traditional therapies in aquaculture (Defoirdt et al., 2007). The potential benefits of using probiotics as a prophylactic tool have gained momentum in the recent decades due to the ability to improve water quality and enhance the nutrient utilization and digestive system (Abdel-Gawad et al., 2021; Sahu et al., 2008; Skjermo & Vadstein, 1999; Verschuere et al., 2000). This leads to better productivity during the culture period. However, the proper application of probiotics to maximize production efficiency together with complementary exogenous enzymes is needed.

A study from Krummenauer et al. (2014) used three replicates to compare the growth of Pacific white shrimp, *L. vannamei*, in a biofloc culture system treated with multispecies probiotics. An increasing trend for growth and survival rate of shrimp was demonstrated, compared with shrimp without any probiotic treatment. In addition, shrimp in the probiotic group also had a lower feed conversion ratio (FCR) compared with the shrimp in the control group. Furthermore, Hamidoghli et al. (2020) also suggested that the administration of a commercial probiotic (AquaStar® Pond, BIOMIN, Austria) can reduce the concentration of total ammonia nitrogen and nitrite in *L. vannamei* culture system. However, novel methods become necessary in attempts to enhance the efficacy of probiotics. Therefore, the aim of the present study was to evaluate the efficacy of multispecies probiotics feeding with enzymes and fermentation process as the common method of applying probiotics in shrimp farm operation on the water quality, growth performance, and protein retention of shrimp *L. vannamei*, cultured intensively in concrete tanks.

2 | MATERIALS AND METHODS

2.1 | Probiotic and enzyme profile

The application method of two different probiotics was investigated in this growth trial. AquaStar® Pond (BIOMIN, Austria) composed of *Bacillus* sp., *Enterococcus* sp., *Pediococcus* sp., *Thiobacillus* sp., and *Paracoccus* sp. (minimum 2×10^{12} CFU kg^{-1}) and AquaStar® PondZyme (BIOMIN, Austria), composed of a similar microbial mix together with enzymes (protease, xylanase, cellulase, and amylase) were used. The products were stored in a cool and dry place until further use. The control treatment was performed using *Bacillus subtilis*, *Lactobacillus* sp., and *Saccharomyces cerevisiae* (Super Lacto, MarindoLab Pratama, Indonesia) minimum 10^6 CFU kg^{-1} fermented with rice bran and enzyme (ß-D-Mannase) for 24 h prior to use in the culture tanks. In this case, no negative control (treatment without probiotics) was employed due to the nature of the commercial trial, where probiotics are an essential part of culture SOPs, especially when high stocking densities are used. The application regime is described in Table 1.

2.2 | Experimental conditions

The study was performed at the Batam Dae Hae Seng Indonesia (Batam, Riau Island, Indonesia). Postlarvae of Pacific white shrimp *Litopenaeus vannamei* (~0.003–0.005 g) were obtained from a commercial hatchery (PT Maju Tambak Sumur, Kalianda, Lampung, Indonesia) and tested negative for white spot syndrome virus (WSSV), Taura syndrome virus (TSV), acute hepatopancreatic necrosis disease (AHPND), Yellow head disease (YHD), and bacterial infection (Fish Quarantine and Inspection Agency, Lampung, Indonesia). Four treatments were carried out with postlarvae (PL) of *L. vannamei* in 24 semi-indoor concrete tanks ($8 \times 8 \times 1$ m) with a stocking density of 500 PL m^2 per tank in a completely randomized design (CRD). The treatments consisted of the addition of 0.2, 0.4, or 0.6 mg L^{-1} of probiotics and enzymes (PE) and compared with 0.6 mg L^{-1} of fermented commercial probiotic (Super Lacto, MarindoLab Pratama, Indonesia) as a control treatment during the first 30 days of the culture period. To produce 1 kg of fermented probiotics, 1 kg of rice bran together with 3–5 g of probiotics, 10 L of culture water, and 3–5 g of enzyme (ß-D-Mannase) were mixed homogenously and cultured fewer than 24 h of aeration period. The addition of probiotics (AquaStar® Pond) into the culture pond was applied every 2 days while the addition of enzymes (AquaStar® PondZyme) were performed every sixth day during the culture period. The addition of fermented probiotic (control) was performed every 2 days throughout the culture period. The doses of PE for all treated tanks were increased by 0.2 mg L^{-1} after 30 days of culture and another 0.2 mg L^{-1} after day 60. The production period was 90 days and the treatments were labeled with (1) 0.2 PE, (2) 0.4 PE, (3) 0.6 PE, and (4) control. The primary source of mechanical aeration was with an air disc fine bubble diffuser, with one 0.5 HP paddlewheel (Minipadd™) per tank providing additional aeration system. Daily water exchanges were 3%–5% throughout the 90 days trial.

2.3 | Feed management

Shrimp in all treatments were fed with the same diet (33%–35% crude protein and 5% crude lipids) produced by Evergreen (Indonesia Evergreen Agriculture, Lampung Selatan, Indonesia) throughout the growth trial. The amount of feed used in this experiment was calculated based on the expected weight gain of 1 g $week^{-1}$, an FCR of 1.4, and a weekly mortality of 3% during the grow-out period. During the trial, shrimp were fed six times per day and the daily ration was adjusted based on the percentage of body weight after sampling the shrimp on a weekly basis.

TABLE 1 The application regime of probiotic (P) and enzyme (E) treated with 0.2, 0.4, and 0.6 PE.

0.2 PE			0.4 PE			0.6 PE		
Day	P	E	Day	P	E	Day	P	E
−2 days	12.8		−2 days	12.8		−2 days	12.8	
0	12.8		0	25.6		0	38.4	
2	12.8		2	25.6		2	38.4	
4	12.8		4	25.6		4	38.4	
6		12.8	6		25.6	6		38.4
8	12.8		8	25.6		8	38.4	
10	12.8		10	25.6		10	38.4	
12		12.8	12		25.6	12		38.4
14	12.8		14	25.6		14	38.4	
16	12.8		16	25.6		16	38.4	
18		12.8	18		25.6	18		38.4
20	12.8		20	25.6		20	38.4	
22	12.8		22	25.6		22	38.4	
24		12.8	24		25.6	24		38.4
26	12.8		26	25.6		26	38.4	
28	12.8		28	25.6		28	38.4	
30		12.8	30		25.6	30		38.4
32	25.6		32	38.4		32	51.2	
34	25.6		34	38.4		34	51.2	
36		25.6	36		38.4	36		51.2
38	25.6		38	38.4		38	51.2	
40	25.6		40	38.4		40	51.2	
42		25.6	42		38.4	42		51.2
44	25.6		44	38.4		44	51.2	
46	25.6		46	38.4		46	51.2	
48		25.6	48		38.4	48		51.2
50	25.6		50	38.4		50	51.2	
52	25.6		52	38.4		52	51.2	
54		25.6	54		38.4	54		51.2
56	25.6		56	38.4		56	51.2	
58	25.6		58	38.4		58	51.2	
60		25.6	60		38.4	60		51.2
62	38.4		62	51.2		62	64.0	
64	38.4		64	51.2		64	64.0	
66		38.4	66		51.2	66		64.0
68	38.4		68	51.2		68	64.0	
70	38.4		70	51.2		70	64.0	
72		38.4	72		51.2	72		64.0
74	38.4		74	51.2		74	64.0	

TABLE 1 (Continued)

0.2 PE			0.4 PE			0.6 PE		
Day	P	E	Day	P	E	Day	P	E
76	38.4		76	51.2		76	64.0	
78		38.4	78		51.2	78		64.0
80	38.4		80	51.2		80	64.0	
82	38.4		82	51.2		82	64.0	
84		38.4	84		51.2	84		64.0
86	38.4		86	51.2		86	64.0	
88	38.4		88	51.2		88	64.0	
90	Harvest		90	Harvest		90	Harvest	

Note: The application for commercial probiotic similar to 0.6 PE treatment.

2.4 | Growth sampling, water quality and total bacteria analysis

Shrimp were sampled weekly throughout the production cycle using a hand net (0.5 m in diameter and 1 cm mesh size) to collect approximately 20–30 individuals per tank. Water quality (DO, pH, temperature, salinity, total dissolved solids, conductivity, and oxidative redox potential) was monitored four times/day (06:00–07:00 a.m., 2:00–3:00 p.m., 5:00–6:00 p.m., and 11:00 p.m.–12.00 a.m.) using real-time water quality sensors (Aqua Troll 500, In-Situ Inc., Fort Collins, CO, USA) and managed by AquaEasy Smart Aquaculture apps (BOSCH, Singapore). Secchi disk readings were recorded once a week. Total ammonia nitrogen (TAN) was analyzed with ultraviolet/visible spectrophotometer (PerkinElmer, Lambda XLS, USA) once a week (Figure 2). Meanwhile, nitrite nitrogen (NO_2—N) and nitrate nitrogen (NO_3—N) were analyzed using HACH DR890 colorimeter (Hach Company, Loveland, CO, USA) twice a week (Figure 2). At the end of the growth trial, shrimp were harvested fully, counted, and batched weighed to calculate the final biomass, final weight, FCR, and survival rate (SR) as shown in Table 2 (Novriadi et al., 2019).

$$\mathrm{FCR} = \frac{\text{feed given (g)}}{\text{alive weigh gain (g)}}.$$

$$\%\mathrm{SR} = \frac{\text{final number of shrimp}}{\text{initial number of shrimp}} {}^{*}100.$$

To count the total number of bacteria in the culture environment, tryptic soy agar (TSA, Difco) was used. To selectively enumerate *Vibrio* spp., thiosulphate-citrate-bile salts-sucrose (TCBS, Difco) agar was used (Ferchs, 1984). Briefly, one milliliter of water sample was serially diluted in autoclaved (121°C for 15 min) seawater to 10^{-4}. Portions (0.1 mL) of each dilution were streaked on the media in duplicates and incubated for 24 h at 35°C. Bacteria and *Vibrio* spp. counts were analyzed weekly for 10 weeks of observation period.

2.5 | Protein retention efficiency

Upon termination of the trial, 4 shrimp from each tank, or 16 shrimp per treatment, were randomly sampled and stored at −60°C for protein retention efficiency analysis. Prior to the protein analysis, dried whole shrimp were

TABLE 2 Growth performance of Pacific white shrimp, *Litopenaeus vannamei* (mean initial weight 0.003–0.005 g), treated with PE for 90 days.

Treatment	Final biomass (kg)	Final mean weight (g)	Survival (%)	FCR	PRE (%)
0.2 PE	242^{ab}	10.3^{c}	78.4^{b}	1.45^{ab}	608^{ab}
0.4 PE	243^{ab}	10.4^{b}	77.7^{b}	1.44^{ab}	621^{ab}
0.6 PE	255^{a}	10.6^{a}	79.9^{ab}	1.37^{b}	629^{a}
Control	236^{b}	9.5^{d}	82.5^{a}	1.48^{a}	556^{b}
PSE	5.3651	0.0514	1.5668	0.0319	24.8102
p-value	<0.0155	<0.0001	0.0271	0.0175	0.0335

Note: Values represent the mean of six replicates. Results in the same columns with different superscript letters are significantly different ($p < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test.
Abbreviations: FCR, feed conversion ratio; PRE, protein retention efficiency; PSE, pooled standard error.

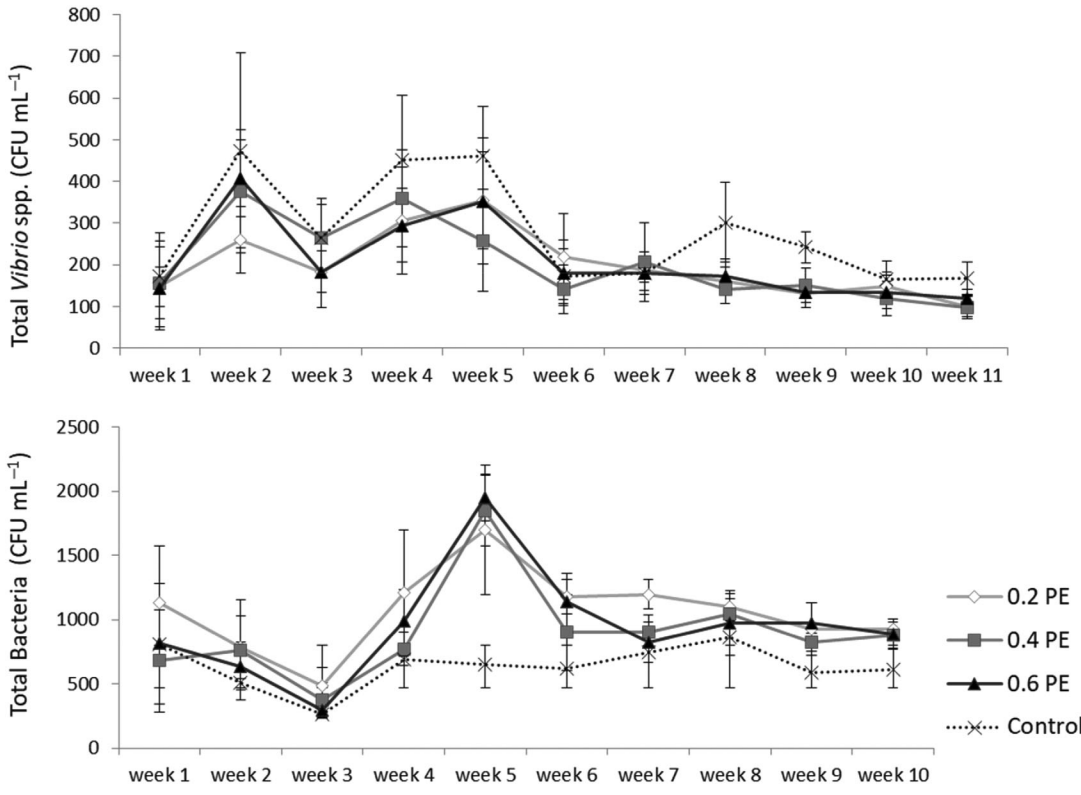

FIGURE 1 Counts of total *Vibrio* spp. and bacteria (CFU mL^{-1}) in the cultured tanks during 70 days of observation period. The four treatments are as follows: (1) 0.2 PE; (2) 0.4 PE; (3) 0.6 PE; and (4) control treatment ($n = 6$).

rigorously blended and chopped in a mixer according to the standard methods established by Association of Official Analytical Chemists (AOAC, 1990). Protein contents of whole shrimp body were analyzed by combustion according to the DUMAS Method (ISO 16634-1; ISO, 2008) and conducted at the Fish Nutrition Laboratory,

Bogor Agricultural University (Bogor, West Java, Indonesia). Calculation for protein retention efficiency (PRE) is displayed in Table 2.

$$\mathrm{PRE} = \frac{(\text{Final weight of shrimp x percent final protein}) - (\text{initial weight x percent initial protein})}{\text{Total protein intake (dry matter)}} * 100.$$

2.6 | Statistical analyses

Growth parameters were analyzed using one-way analysis of variance (ANOVA) to determine significant differences among treatments followed by Tukey's multiple comparison tests to determine the difference between treatment means in each trial using SAS system (V9.4. SAS Institute, Cary, NC, USA). Water quality data were analyzed in a linear mixed effects model with treatment, dose, and time as fixed effects and tank nested in time as random effects to account for the nature of repeated measurements using RStudio, package "nlme" (RStudio 2022.02.0+443 Windows).

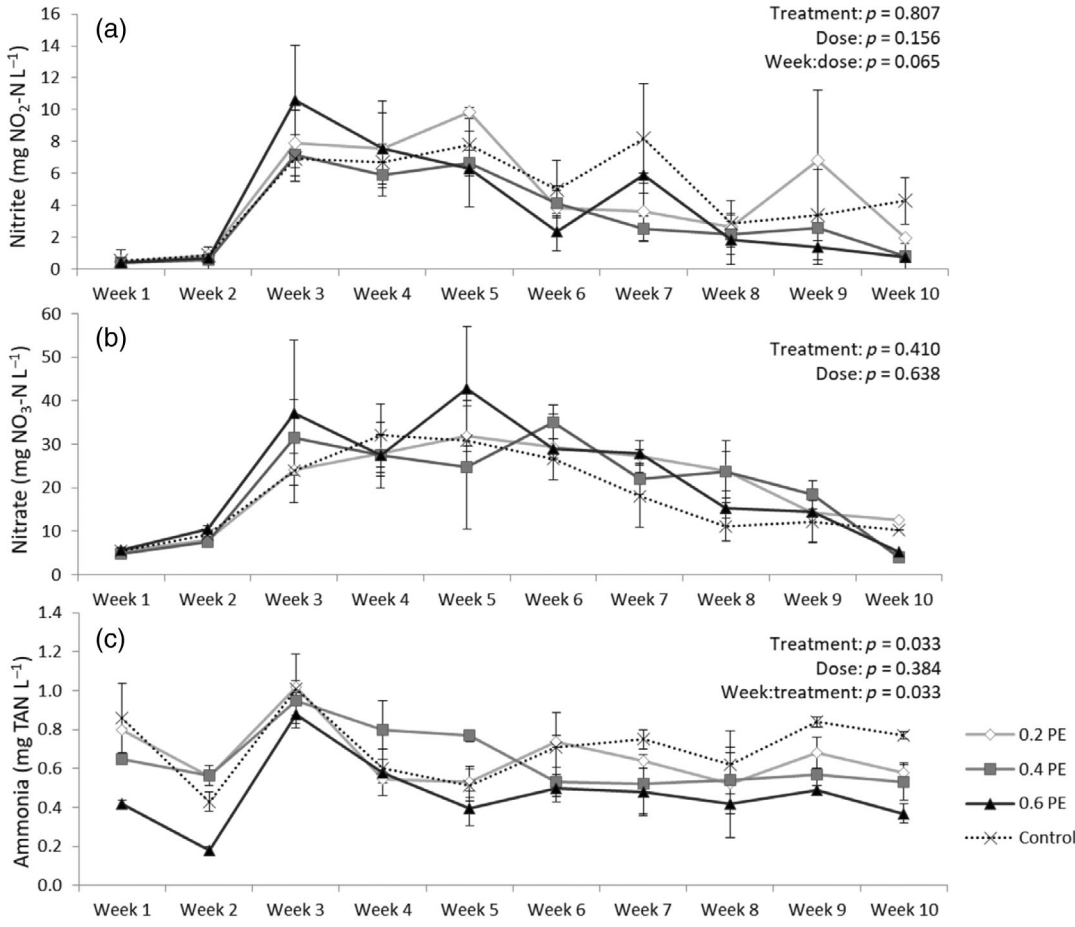

FIGURE 2 Concentration of (a) nitrite (mg NO_2—N L^{-1}); (B) nitrate (mg NO_3—N L^{-1}); and (C) Total ammonia (mg TAN L^{-1}) treated with four different treatments. Namely: (1) 0.2 PE, (2) 0.4 PE, (3) 0.6 PE, and (4) control treatment during 70 days of observation period

3 | RESULTS

3.1 | Total bacteria and *Vibrio* spp. count

The results for total bacterial count (TBC) and total bacterial *Vibrio* spp. (TBV) in the culture environment for the trial period are shown in Figure 1. In general, counts of total *Vibrio* spp. (CFU mL^{-1}) in the group of shrimp treated with fermented probiotic (control treatment) had a slight upward trend compared with the group of shrimp treated with multispecies probiotics and enzyme (PE), albeit not significant. Meanwhile for TBC (CFU mL^{-1}), the group of PE treatment showed a peak in the number of bacteria compared with the control treatment around Week 5.

3.2 | Water quality and growth performance

Based on the online data recording system, pH, water temperature (°C), and dissolved oxygen (mg L^{-1}) were in the range of 7.72 ± 0.12, 29.33 ± 1.92°C, and 5.91 ± 0.31 mg L^{-1} for group 0.2 PE; 7.83 ± 0.08, 29.46 ± 1.69°C, and 5.89 ± 0.22 mg L^{-1} for group of 0.4 PE; 7.88 ± 0.11, 29.44 ± 1.55°C, and 5.93 ± 0.17 mg L^{-1} for group of 0.6 PE; and 7.58 ± 0.14, 29.29 ± 1.88°C, and 5.88 ± 0.24 mg L^{-1} for the group of control treatment. Meanwhile, TAN, NO_2—N, and NO_3—N peaked from weeks 3–5 and declined slowly until the end of the observation period (Figure 2). In terms of TAN, from the seventh week of the trial, the concentration levels within the PE groups were always lower compared with the control treatment, resulting in an overall significant difference to control ($p = 0.033$).

At the end of the culture period, growth performance differed significantly ($p < 0.05$) between the commercial probiotic and enzyme treatment and control group (Table 1). The group of 0.6 PE had 8% higher final biomass and 11% higher final mean weight compared with the control treatment at the end of the culture period. FCR in the group of 0.6 PE was by 7.4% lower compared with the control treatment, PRE (%) was by 13% higher in shrimp treated with 0.6 PE compared with the control treatment. The optimization of feed utilization in this group was achieved by using a quantified dosage of PE, compared with the common practices of fermented probiotics application in Indonesia.

4 | DISCUSSION

The present study demonstrates the effectiveness of the commercial probiotics, containing *Bacillus* sp., *Enterococcus* sp., *Pediococcus* sp., *Thiobacillus* sp., and *Paracoccus* sp., in combination with an enzyme cocktail (PE) to enhance the growth of shrimp and productivity of the culture environment. This was an attempt to evaluate the efficacy of continuous application of PE in comparison with common application methods of fermented probiotics in intensive farming system.

The results of this study showed that continuous application of multispecies PEs could reduce the amount of total *Vibrio* spp. in the culture environments. It is well documented that probiotics can inhibit the growth of a wide variety of opportunistic bacteria, such as *Vibrio* spp. and reduce the prevalence of viruses (Fu et al., 2011; Joseph et al., 2013). According to Dalmin et al. (2001), Moriarty (1997) and Kewcharoen and Srisapoome (2019), Bacilli are one group of probiotics that could inhibit the growth of pathogenic *Vibrio* spp. by competing for nutrients, and damaging the slime layers of *Vibrio* spp. through enzyme secretions. Besides *Bacillus* spp., the other probiotic species used in this study also, including lactic acid bacteria (LAB), are well-known bio-controllers. Several researchers have demonstrated that the use of LAB showed strong immunomodulatory functions and capacity to inhibit the growth of *Vibrio* spp. (Chomwong et al., 2018; Nguyen Thi Truc et al., 2019; Sha et al., 2016; Vieira et al., 2007). Therefore, the addition of exogenous enzyme in probiotic application process can also work synergistically in improving the growth performance and health status of *L. vannamei* (Maas et al., 2021).

As a general tendency, the level of total TAN, NO_2-N, and NO_3-N had lower numerical value in the group of shrimp treated with continuous application of PE compared with control treatment. There are many reports on the use of probiotic products to enhance the water quality conditions and increase the removal rate of ammonia (Farzanfar, 2006). According to Shariff et al. (2001), bacterial species belonging to the genera *Bacillus*, *Pseudomonas*, *Nitrosomans*, *Nitrobacter*, *Acinetobacter*, and *Cellulomonas* are known to help in the mineralization of organic water and in reducing the accumulation of organic loads. Probiotics could also enhance the decomposition of organic matter, reduce nitrogen and phosphorus concentrations, and control the level of ammonia, nitrite, and hydrogen sulfide (Boyd & Massaut, 1999) and enhance the environmental conditions of the production system (Suhendra et al., 1997). Much of these data comes from in vitro or small-scale animal trials. It should be noted that this study was carried out in 64 m^3 concrete tanks, and therefore the results could provide useful information about the efficacy of PE to increase the productivity in commercial intensive systems.

The stocking density used in this growth trial was 500 PL m^2 and specified as an intensive scale of shrimp culture system (AQUACOPs, 1984). In this type of culture system, applying appropriate management strategies are essential to ensure the optimization of feed utilization, which also affects the farm productivity, FCR, growth rate, water pollution, and economic returns of the culture system (Van et al., 2017). In this growth study, the 0.6 PE group resulted in greater biomass compared with the control group. In terms of quality, the mean weight of the individual shrimp at the end of the growth trial was significantly higher in all groups treated with PE compared in the control group. This fact is even more remarkable when taking into account the lower FCR and the highest PRE in the group of shrimp treated with PE compared with the control group. These results could be associated with the positive effect of probiotic bacteria, which act as a supporting element to the dynamic equilibrium of the intestinal microflora, thereby reducing digestive problems and providing high levels of enzymatic activity, as well as increasing digestive function (Wang, 2007). Since the shrimp digestive system is activated particularly in larval and early post-larval stages, the use of additional protease, xylanase, cellulase, and amylase enzymes in this study might have a synergistic effect, which might in turn explain the better growth, protein composition (%), and PRE (%) of shrimp treated with PE during the culture period.

5 | CONCLUSION

Under such conditions, the continuous application of commercial PEs significantly increased the growth performance of shrimp compared with the control group. Water quality conditions in the concrete tank culture system, especially for the number of *Vibrio* sp., level of TAN, NO_2-N, and NO_3-N, were better in the tanks treated with PEs compared with the control group. Despite many variations within the treatments, biologically, the combination of PEs could enhance the nutrient utilization as shown in the protein composition (%) of the whole body of shrimp. Based on these results, for intensive shrimp culture systems, multispecies PEs should be applied at 0.6 mg L^{-1} during the first 30 days, 0.8 mg L^{-1} until day 60, and 1.0 mg L^{-1} until harvest is encouraged to stimulate better productivity, controlling *Vibrio* spp., and maintaining water quality conditions in intensive culture environments.

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CONFLICT OF INTEREST

Jutta Kesselring and Benedict Standen are employed by BIOMIN Holding GmbH. The rest of the authors state no conflict of interest.

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