Hindawi
Aquaculture Nutrition
Volume 2022, Article ID 1861761, 13 pages
https://doi.org/10.1155/2022/1861761

Research Article

Effects of Dietary Pectin and *Lactobacillus salivarius* ATCC 11741 on Growth Performance, Immunocompetence, Gut Microbiota, Antioxidant Capacity, and Disease Resistance in Narrow-Clawed Crayfish, *Postantacus leptodactylus*

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Received 18 July 2022; Revised 14 September 2022; Accepted 9 October 2022; Published 2 November 2022

Academic Editor: Erchao Li

The present study was conducted to clarify the effects of *Lactobacillus salivarius* (LS) ATCC 11741 and pectin (PE) on growth performance, digestive enzymes activity, gut microbiota composition, immune parameters, antioxidant defense as well as disease resistance against *Aeromonas hydrophila* in narrow-clawed crayfish, *Postantacus leptodactylus*. During 18 weeks trial feeding, 525 narrow-clawed crayfish juvenile (8.07 ± 0.1 g) fed with seven experimental diets including control (basal diet), LS1 (1×10^7 CFU/g), LS2 (1×109 CFU/g), PE1 (5 g/kg), PE2 (10 g/kg), LS1PE1 (1×10^7 CFU/g +5 g/kg), and LS2PE2 (1×109 CFU/g +10 g/kg). After 18 weeks, growth parameters (final weight, weight gain, and specific growth rate) and feed conversion rate were significantly improved in all treatments ($P < 0.05$). Besides, diets incorporated with LS1PE1 and LS2PE2 significantly increased the activity of amylase and protease enzymes compared to LS1, LS2, and control groups ($P < 0.05$). Microbiological analyses revealed that the total heterotrophic bacteria count (TVC) and lactic acid bacteria (LAB) of narrow-clawed crayfish fed diets containing LS1, LS2, LS1PE1, and LS2PE2 were higher than control group. The highest total haemocyte count (THC), large-granular (LGC) and semigranular cells (SGC) count, and hyaline count (HC) was obtained in LS1PE1 ($P < 0.05$). Similarly, higher immunity activity (lysozyme (LYZ), phenoloxidase (PO), nitroxidesynthetase (NOs), and alkaline phosphatase (AKP)) observed in the LS1PE1 treatment compared to the control group ($P < 0.05$). The glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity remarkably enhanced in LS1PE1 and LS2PE2, while malondialdehyde (MDA) content reduced in these two treatments. In addition, specimens belonging to LS1, LS2, PE2, LS1PE1,

and LS2PE2 groups presented higher resistance against *A. hydrophila* compared to the control group. In conclusion, feeding narrow-clawed crayfish with synbiotic had higher efficiency on growth parameters, immunocompetence, and disease resistance compared to single consumption of prebiotics and probiotics.

1. Introduction

Employing novel protein sources can reduce the risk of global food shortages due to war, drought, and unfair food distribution [1]. In this regard, narrow-clawed crayfish (*Postantacus leptodactylus*) is a high-quality aquaprotein that could help to address protein deficiency and global food insecurity [2–4]. In recent years, the reduction of the wild crayfish population, the limitation of settling in other resources as well as the high nutritional and commercial value have increased the focus on crayfish production under captivity conditions [5, 6]. However, astaciculture is challenged by various problems such as a lower growth rate than tropical shrimp under cultured conditions, infectious diseases, and environmental stresses [2, 7].

Studies on functional compounds like probiotics, prebiotics, and synbiotics that may enhance growth rate, immune responses, antioxidant defenses, and subsequently disease resistance in narrow-clawed crayfish have become a promising strategy in aquaculture [8].

Synbiotics are the combination of live microbial adjuncts (probiotics) and indigestible compounds (prebiotics) that beneficially affect the physiological activities of commercial species during the rearing period [9, 10]. The use of synbiotics is an effective way to induce immunoregulation in aquatic animals, which in turn increases the disease resistance to infectious agents [11, 12]. On the other hand, synbiotics are also known as growth promoters that can cover some production costs [13, 14]. Other findings showed that diets containing synbiotics improve the resistance of fish against stressful events through the scavenging of reactive oxygen species (ROS) and altering the activity of antioxidant enzymes [15]. In astaciculture, Safari and Paolucci [8] also reported that galactooligosaccharide + *Enterococcus faecalis* in narrow-clawed crayfish were found to trigger growth performance, innate immune responses, and antioxidant enzyme activities.

Lactobacillus salivarius is a gram-positive bacterium that has been isolated from the breast milk and the cecum of animals [16, 17]. So far, several studies have demonstrated the probiotic properties of *L. salivarius* including the production of natural antibiotics and short chain fatty acids, the reduction of gut pH, and the modulation of gut microbiota [16, 18]. In animals, *L. salivarius* in the chicken diet improved growth performance, boosted immune responses, and attenuated the negative effects of stress [19]. Another study demonstrated that the colonization of *L. salivarius* in the gut of rats led to improved raffinose breakdown, nutrient absorption, and immune responses [20]. Despite the potential benefits of *L. salivarius*, there is no available information on the possible impacts of *L. salivarius* on aquatic animals.

Pectin is a natural prebiotic that is widely obtained from the skins and wastes of some fruits, such as apple and kiwifruit pomace, citrus, papaya, and banana peels, which is employed as a low-cost carbon source for probiotics [21, 22]. Pharmacological findings have proven the therapeutic properties of pectin including lowering plasma cholesterol, against senescent, and anticancer and antidiarrhoeal properties in humans [23, 24]. Furthermore, the recent findings revealed that low molecular weight and degree of methylation were useful for a wide range of probiotics [25]. Gómez et al. [26] reported that pectin in vitro increased joint populations of bifidobacteria and lactobacilli from 19% to 34% and 29%, respectively. In aquaculture, dietary pectin also has revealed beneficial effects on improved growth performance, innate immune responses, and disease resistance in different hosts such as Nile tilapia *Oreochromis niloticus* [22], zebrafish, *Danio rerio* [27] as well as antioxidant defense in common carp, *Cyprinus carpio* [28, 29]. Besides, trials carried out by Kuo et al. [30] showed that a combination of *Lactobacillus plantarum* and pectin as carbon source provided a notable improvement in growth performance and immunocompetence in *Litopenaeus vannamei*. It seems that the combination of pectin and *L. salivarius* affected the physiological processes of the host more effectively than the single form of pro-/prebiotic, due to possible synergistic effects. Due to the increasing demand for narrow-clawed crayfish, the use of potential synbiotics can support *P. leptodactylus* production. Therefore, the present study was conducted to investigate the effects of pectin and *L. salivarius* in single and combined (synbiotic) forms on growth performance, immunohaemocyte, gut microbiota profile, antioxidant capacity, and disease resistance in narrow-clawed crayfish, *P. leptodactylus*.

2. Material and Methods

2.1. Preparation of the Experimental Diet. In this work, pectin was purchased from Sigma-Aldrich Inc. (P9135, obtained from citrus peel, galacturonic acid ≥ 74.0%). *L. salivarius* ATCC 11741 (IBRC-M 10865) was prepared from the Iranian Biological Resource Center (Tehran, Iran). The bacterium was cultured in Man Rogson Sharp (MRS; Merck, Germany) at 37°C for two overnight, then centrifuged at 4000 g for 20 minutes and washed twice with phosphate-buffered saline solution (PBS; pH 7.2). Afterward, it was suspended in PBS, and the desired concentrations including 1×10^7 and 1×10^9 CFU/g were adjusted. In this work, a basal diet was formulated based on Safari and Paolucci [8]. The feedstuffs used to prepare the experimental diets along with the biochemical composition of each diet are presented in Table 1. In summary, the powdered food ingredients were mixed well together (20 min), and then the homogenized mixture was pasted using water. Then, the pellets with a diameter of 2 mm were produced via an industrial meat grinder (National Meat Grinder MK-G20NR, Japan). The obtained pellets were stored at room temperature for 24 h, and stored in plastic bags in the refrigerator. Other experimental diets were made by adding dietary supplements including LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L.*

Table 1: Feedstuff and proximate analysis of the experimental diets.

Ingredients	Diets (g/kg in dry basis)						
	Control	LS1	LS2	PE1	PE2	LS1PE1	LS2PE2
Fishmeal[a]	147	147	147	147	147	147	147
Wheat flour[b]	289	289	289	289	289	289	289
Soybean meal[b]	175	175	175	175	175	175	175
Corn gluten[b]	112	112	112	112	112	112	112
Starch meal[b]	49	49	49	49	49	49	49
Soybean oil[b]	41	41	41	41	41	41	41
Lectin[b]	50	50	50	50	50	50	50
Fish oil[b]	42	42	42	42	42	42	42
Cholesterol[c]	5	5	5	5	5	5	5
Glucosamine[d]	10	10	10	10	10	10	10
Choline chloride[c]	15	15	15	15	15	15	15
Carboxymethyl cellulose[d]	19.9	19.9	19.9	14.9	9.9	14.9	9.9
Ytterbium oxide[d]	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin C[c]	10	10	10	10	10	10	10
Vitamin premix[e]	20	20	20	20	20	20	20
Mineral premix[e]	15	15	15	15	15	15	15
L. salivarius (CFU/g)		1×10^7	1×10^9			1×10^7	1×10^9
Pectin (g)	0	0	0	5	10	5	10
Chemical analysis of the experimental diets (g/kg dry matter basis)							
(i) Dry matter	872	872.02	872.03	877.20	883.3	877.22	883.33
(ii) Crude protein	382.3	382.3	382.3	382.65	382.95	382.65	382.95
(iii) Crude lipid	127	127	127	1271.15	127.32	127.15	127.32
(iv) Ash	37.9	37.92	37.93	36.60	36.90	36.62	36.93
(v) Fiber	30.4	30.40	30.4	32.7	35.4	32.70	35.5
(vi) NFE[f]	422.4	422.38	422.37	420.9	417.43	420.88	417.3

[a]Peygir Co., Gorgan, Iran. [b]Behparvar Aquafeed Co., Iran. [c]Kimia Roshd Co., Iran. [d]Sigma, Germany. [e]The premix provided the following amounts per kg of feed: A:1000 IU; D3: 5000 IU; E: 20 mg; B5: 100 mg; B2: 20 mg; B6: 20 mg; B1: 20 mg; H: 1 mg; B9: 6 mg; B12: 1 mg; B4: 600 mg; C:50 mg; Mg: 350 mg; Fe: 13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg; Se: 0.3 mg; I: 1.5 mg; and Mn: 10 mg. Chinechin Co., Tehran, Iran [c]Kimia Roshd Co., Iran. [f]Nitrogen-free extracts (NFE) = 1000–(crude protein + crude lipid + ash + fiber).

salivarius, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*; 1×10^7 CFU/g + Pectin, 5 g/kg) and LS2PE2 (*L. salivarius*, 1×10^9 CFU/g + Pectin, 10 g/kg), to the dough. Different levels of supplements in this study were selected based on the positive results of previous reports of pectin and other lactic acid bacteria on fish and shellfish [6, 22, 31, 32]. Experimental diets were prepared biweekly to ensure that high *L. salivarius* values remained in the feeds for the duration of the trial [33]. Survival and number of colonies per each diet were confirmed using culture in tryptic soy agar (TSA; Merck, Germany) culture medium. This process was done twice a week [34] and the mean concentration of *L. salivarius* in supplemented diets during two weeks was based on CFU/g as follows: LS1: 0.88×10^7, LS2: 0.87×10^9, LS1PE1: 0.80×10^7, and LS2PE2: 0.75×10^9.

2.2. Experimental Procedure. In this work, five hundred and fifty narrow-clawed crayfish juveniles with an average weight of (7 ± 0.20 g, mean ± SD) were collected from Aras Dam Lake (West Azerbaijan, Iran.) and transferred to a private farm in Rasht City (Iran). Adaptation was performed for 14 days in fiberglass tanks (1000 L) at DO: 6.5 ± 0.20 mg/L, pH: 7.28 ± 0.34, hardness: 143 ± 5.5 mg/L $CaCO_3$, unionized ammonia (<0.05 mg/L), temperature: $23 \pm 1^{\circ}$C, and specimens were fed with the basal diet, four times a day (7:00, 11:00, 16:00, and 21:00). This work was carried out in 7 experimental groups with 3 replications. For this, five hundred and twenty five narrow-clawed crayfish (8.07 ± 0.1 g, mean ± SD) were divided into 21 cubic polyethylene tanks ($60 \times 44 \times 160$ cm) at a density of 25 specimens per each tank. Animals belonging to the control group fed with the basal diet and other experimental groups received diets supplemented with probiotics or pectin including LS1, LS2, PE1, PE2, LS1PE1, and LS2PE2 four times a day based on apparent satiety for 18 weeks (July 2018 to December 2018). To calculate the eaten food, four hours after each meal, the unconsumed food was collected from each tank and dried in oven at 60. To prevent aggressive behaviors, plastic tubes (diameter: 4 cm and length: 11 cm) were placed in each tank. The rearing system was without water flow (static) and water quality was maintained by continuous aeration, removing

uneaten food and particulate matter, and changing 30% of the water, daily. Also, physicochemical parameters were checked once a week.

2.3. Zootechnical Parameters and Feed Utilization. At the end of the feeding trial, the total biomass of each tank was weighed. During the rearing period, uneaten food was collected from each tank and dried. Finally, growth and nutritional parameters and survival rate were estimated based on the following formulas [35]:

$$\begin{aligned}
&\text{Weight gain (WG ; g)} = \text{mean final weight (g)} - \text{mean initial weight (g)},\\
&\text{Specific growth rate (SGR ; \%/day)} = \text{Ln [mean final weight (g)]} - \text{Ln [mean initial weight (g)]} \times \frac{100}{\text{days}},\\
&\text{Feed conversion ratio (FCR)} = \text{feed intake} \frac{(\text{g})}{\text{weight}} \text{gain (g)},\\
&\text{Survival rate (SR, \%)} = (\text{Nf}/\text{Ni}) \times 100.
\end{aligned} \tag{1}$$

Nf is the narrow-clawed crayfish number at final of trial feeding and Ni is the narrow-clawed crayfish number at initial of trial feeding.

2.4. Quantification of Digestive Enzymes. At the end of the 18th week, all animals in each tank were fasted for 24 hours and three crayfish were harvested from each replicate and dissected on ice plates. In the next step, the hepatopancreatic tissue was isolated, washed with distilled water, and gently dried using a towel. Afterward, samples were mixed with Tris-HCl buffer (W/V, 50 mM, pH: 7.0) and homogenized via an electric homogenizer (D 500). The homogenized mixture was centrifuged at 10000 g at 4°C for 25 min (Hermle Z36HK, Germany), and the supernatant was aliquoted and stored at −70°C [36]. Alpha-amylase activity was measured using starch as a substrate (0.3%) diluted in Na_2HPO_4 buffer (pH: 7.4). In this method, the reaction was stopped via dinitrosalicylic acid reagent, and the absorbance was recorded at 540 nm [37]. The level of alkaline protease activity was determined using 2% azo-casein solution in 50 mM Tris-HCl (pH: 9.0) as substrate. After incubation of the mixture at 25°C for 10 min, the reaction was stopped by 0.5 mL TCA (Trichloroacetic Acid). The samples were centrifuged at 6500 g for 5 min and the absorbance was recorded at 440 nm [38]. Lipase activity was measured based on the method described by Iijima et al. [39] and using p-nitrophenol myristate (Sigma N2502) as the substrate dissolved in 0.25 mM Tris-HCl (pH: 9.0) along with 0.25 mM 2-methoxyethanol and 5 mM sodium cholate solution. The reaction was terminated by the addition 0.7 mL of acetone/heptane (5 : 2 v/v), and the mixture was centrifuged (6000 g at 4°C for 5 min) and the absorbance of the supernatant was recorded at 405 nm.

2.5. Bacteriological Assay. To evaluate the gut microbiota, the skin surface of the individual was washed using distilled water and then disinfected with ethanol 70%. Thereafter, the animals were dissected aseptically using a sterile instrument, the gut was isolated, washed with PBS, and homogenized using an electric homogenizer (DI 18 Disperser). The obtained homogeneity was continuously diluted in PBS (pH: 7.2). In the next step, 100 μL of the sample was spread onto MRS and plate count agar (PCA; Merck Co) for the determination of lactic acid bacteria (LAB) and total viable heterotrophic bacteria count (TVC), respectively. The pellets were saved at 28°C for 48 h and TVC and LAB colonies were counted and reported in tissue CFU/g [40].

2.6. Hemolymph Collection. At the end of the 18th week, three starved narrow-clawed crayfish were caught from each tank, and hemolymph was drawn from the ventral sinus using a syringe with a needle 25 g. The obtained hemolymph was stored for two targets. One part was placed in a 2 mL Eppendorf containing Alsever buffer as anticoagulant agent (115 mmoL glucose, 336 mmoL NaCl, 27 mM sodium citrate, and 9 mM EDTA with pH: 7.0). 200 μL of a hemolymph-anticoagulant sample was used to estimate total haemocyte count (THC) and differential counts of haemocytes (DHC). The remaining part was immediately centrifuged at 1000 g for 5 min at 4°C and the supernatant was stored to assess immunity indicators [8].

2.6.1. Hemolymph Indices. THC (cells/mL) was counted via a hemocytometer (Neubauer, Germany) under a light microscope [41]. Assay DHC (cells/mL) was carried out by preparing the hemolymph smear, complete drying of smears exposed to airflow, fixing in 70% methanol for 10 min, and staining based on May-Grunwald and Giemsa technique [42]. Finally, the number of large granular, semigranular cell granular, and the hyaline cell was counted using a light microscope (Nikon E100; Nikon, Tokyo, Japan).

2.6.2. Hemolymph Immune Parameters. Plasma lysozyme (LYZ) activity was measured based on plasma capability in lysis of *Micrococcus luteus* using turbidity test [43], and recording OD at 450 nm. Phenoloxidase (PO) activity was determined spectrophotometrically (Pharmacia Biotech Ultrospec 2000) via the production of dopachrome from L-dihydroxy phenylalanine (LDOPA, Sigma), and reading OD at 490 nm [44]. Nitric oxide synthase (NOS) activity rate was calculated by the commercially available kit (Nanjing Jiancheng Bioengineering Institute, China) [45]. The activities of alkaline phosphatase (AKP) and acid phosphatase (ACP) were detected using the method described by Hao et al. [46]. In this method, disodium phenyl phosphate-4-aminoantipyrine-potassium ferricyanide and disodium phenyl phosphate were used as the substrate to estimate the activity of AKP and ACP, respectively.

2.6.3. Hemolymph Antioxidant Enzymes Activities and Malondialdehyde Content. Superoxide dismutase activity was estimated by a commercial kit (ZellBio GmbH, Germany) based on the protocol supplied by the manufacturer. In this method, 10 μL hemolymph supernatant was transferred to each well of the microplate (96-well). Afterward, 250 μL reagent 1, 10 μL reagent 2, and 10 μL distilled water were poured into each chamber. In the next step, the chromogenic matter was added to the related chambers. Finally, the color change was recorded with a microplate reader at 420 nm [6]. Catalase activity was detected using a commercial kit (ZellBio GmbH, Germany), which was used previously for crayfish [47]. The OD was estimated colorimetrically via the microplate reader at a wavelength

of 405 nm. The glutathione peroxidase (GPx) was detected by quantifying the level of H_2O_2 in the presence of glutathione (GSH), according to the protocols recommended by the manufacturer (ZellBio GmbH, Germany). Malondialdehyde (MDA) content was estimated by reaction of thiobarbituric with malondialdehyde and maximum OD was recorded at 534 nm [47].

2.7. In Vivo Crayfish Infection Test. At the end of the feeding trial, the challenge was performed based on complete hygiene and quarantine principles. *Aeromonas hydrophila* (AH04) was purchased from the Faculty of Veterinary Medicine, University of Tehran. Stocks were grown on TSA medium at 37°C for 24 h. The grown bacteria were centrifuged at 10,000 × g and 4°C for 10 min. Pellets were washed twice using PBS. The desired dose was adjusted at 1×10^8 cells/mL using sequential dilution and based on previous results [8]. Crayfish were infected by injecting 20 μL into the ventral sinus [48, 49]. The injected specimens (10 samples per aquarium) were transferred to a glass aquaria (V: 100 L water). The challenge period lasted for 5 days and infected animals were checked for mortality rate (MR).

2.8. Statistical Analysis. In this study, data analysis was carried out using SPSS software (version 22, (SPSS Inc., Chicago, USA). In the first step, homogeneity of variance and normality of the data were confirmed using Leaven's and Kolmogorov-Smirnov tests, respectively. In the next step, differences between the results of experimental groups were evaluated via one-way ANOVA analysis. Besides, significant differences between the treatments were determined using Tukey's test. The results were presented as mean ± SD.

3. Results

3.1. Growth Performance. The growth performance of *P. leptodactylus* fed with levels of LS and PE on the 18th week is indicated in Table 2. On the 18th week, the treated diets included LS, PE, and LSPE showed a significant increase in growth indices including FW, WG, and SGR compared to the control group ($P < 0.05$). Besides, the highest FW, WG, and SGR were obtained in animals fed with the diet containing LS1PE1, which showed a significant difference with those receiving diets LS1, LS2, and basal diet ($P < 0.05$). In contrast, an inverse pattern was revealed in FCR among different experimental groups. In this regard, the highest FCR was recorded in crayfish fed the control diet, which presented a significant difference compared to the treated groups ($P < 0.05$). Among the experimental groups, the lowest FCR was recorded in fish receiving the LS1PE1 diet. SR was also statistically similar among the experimental groups ($P > 0.05$).

3.2. Digest Enzymes Activities. The digestive enzyme activities of the narrow-clawed crayfish after 18 weeks of feeding are presented in Figure 1. Feeding animals with diets incorporated with LS1PE1 and LS2PE2 significantly improved the activity of amylase and protease enzymes compared to ones fed with LS1, LS2, and basal diets ($P < 0.05$). However, lipase activity was not affected by supplemented diets ($P > 0.05$).

3.3. Microbiological Analysis. The microbiota composition of narrow-clawed crayfish after the 18 weeks feeding trial is shown in Figure 2. The highest TVC was obtained in the gut of animals fed with diets containing LS1, LS2, LS1PE1, and LS2PE2 in comparison with crayfish belonging to PE1, PE2, and control groups ($P < 0.05$). Moreover, crayfish fed with feeds containing LS1PE1, and LS2PE2 showed higher LAB counts compared to animals that received PE1, PE2, and basal diets ($P < 0.05$).

3.4. Hemolymph Indices. As indicated in Table 3, hemolymph indices in groups fed with different levels of probiotic, prebiotic, and synbiotic were significantly affected ($P < 0.05$). The THC count in response to the diet containing LS1PE1 indicated a significant difference compared to other groups, except for LS2PE2 ($P < 0.05$). HC count in all groups fed with probiotic, prebiotic, and synbiotic was significantly higher than narrow-clawed crayfish which belongs to the control group ($P < 0.05$). Also, feeding crayfish with LS1PE1 and LS2PE2 diets remarkably increased LGC compared to PE1, PE2, and control groups ($P < 0.05$). Administration of LS1, LS1PE1, and LS1PE1 resulted in a remarkable enhancement in SGC count in compared to PE1 and PE2, and control groups ($P < 0.05$).

3.5. Immunological Responses. The effect of the lactobacillus, pectin, and synbiotic on immunity responses of narrow-clawed crayfish at the end of 18th week is exhibited in Table 4. Administration LS1PE1 significantly increased LYZ and PO activities compared to the control group ($P < 0.05$). Additionally, a peak NOS activity was recorded in crayfish fed with diets supplemented with PE1, PE2, LS1PE1, and LS2PE2 in comparison with the control group ($P < 0.05$). AKP activity in all treatments was remarkably elevated compared to control groups ($P < 0.05$). However, there was no significant difference in ACP between different treatments and the control group ($P > 0.05$).

3.6. Antioxidant Defense. Alterations in the level of crayfish antioxidant enzymes in response to different levels of probiotics, pectin, and synbiotic are presented in Figure 3. SOD activity of narrow-clawed crayfish in LS1PE1 and LS2PE2 treatments was higher than PE2 and control groups ($P < 0.05$). GPx activity was remarkably increased in all treatments compared to the control group ($P < 0.05$). However, the level of CAT activity in experimental treatments was statistically similar ($P > 0.05$). The lowest MDA value was recorded in both LS1PE1 and LS2PE2 treatments.

3.7. Disease Resistance. The mortality rates of crayfish infected with *Aeromonas hydrophila* are shown in Figure 4. Accordingly, the percentage of mortality rate (MR) in crayfish belonging to the control group was higher than those of the other groups on different days. At the end of the challenge period, the highest and lowest MR were recorded in the control (66.66%) and LS1PE1 (23.33%) groups, respectively. Experimental diets containing LS1, LS2, PE2, LS1PE1, and LS2PE2 significantly increased disease resistance compared to the control group.

Table 2: Growth performance indices of narrow-clawed crayfish (*Postantacus leptodactylus*) fed with basal diet and supplemented with LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L. salivarius*, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*, 1×10^7 CFU/g + Pectin, 5 g/kg) and LS2PE2 (*L. salivarius*, 1×109 CFU/g + Pectin, 10 g/kg) for 18 weeks.

	Treatments						
Parameters	Control	LS1	LS2	PE1	PE2	LS1PE1	LS2PE2
IW(g)	8.11 ± 0.07^{a}	8.13 ± 0.12^{a}	8.15 ± 0.8^{a}	7.96 ± 0.07^{a}	8.10 ± 0.10^{a}	8.05 ± 0.09^{a}	8.01 ± 0.13^{a}
FW (g)	26.00 ± 1.50^{d}	31.83 ± 1.75^{c}	32.93 ± 2.00^{bc}	36.83 ± 2.56^{abc}	36.36 ± 2.12^{abc}	40.93 ± 1.90^{a}	38.16 ± 2.25^{ab}
WG (g)	17.88 ± 1.57^{d}	23.70 ± 1.81^{c}	24.79 ± 2.07^{bc}	28.86 ± 2.49^{abc}	28.26 ± 2.14^{abc}	32.89 ± 1.91^{a}	30.15 ± 2.26^{ab}
SGR (%/d)	0.92 ± 0.05^{d}	1.08 ± 0.06^{c}	1.10 ± 0.07^{bc}	1.21 ± 0.04^{abc}	1.19 ± 0.05^{abc}	1.29 ± 0.03^{a}	1.23 ± 0.04^{ab}
FCR	3.90 ± 0.32^{a}	2.98 ± 0.22^{b}	2.78 ± 0.19^{bc}	2.40 ± 0.21^{bcd}	2.46 ± 0.19^{bcd}	2.11 ± 0.15^{d}	2.30 ± 0.20^{cd}
SR (%)	77.33 ± 2.30^{a}	80 ± 4.00^{a}	78.66 ± 2.30^{a}	82 ± 2.30^{a}	80.25 ± 6.92^{a}	85.33 ± 2.30^{a}	81.33 ± 2.40^{a}

Note: Different letters in each column show significant differences among the experimental groups ($P < 0.05$). Values are shown as mean ± SD ($n = 3$). Abbreviations: IW: initial weight (g); FW: final weight (g); WG: weight gain (g); SGR: specific growth rate (%/d); FCR: feed conversation rate; SR: survival rate (%).

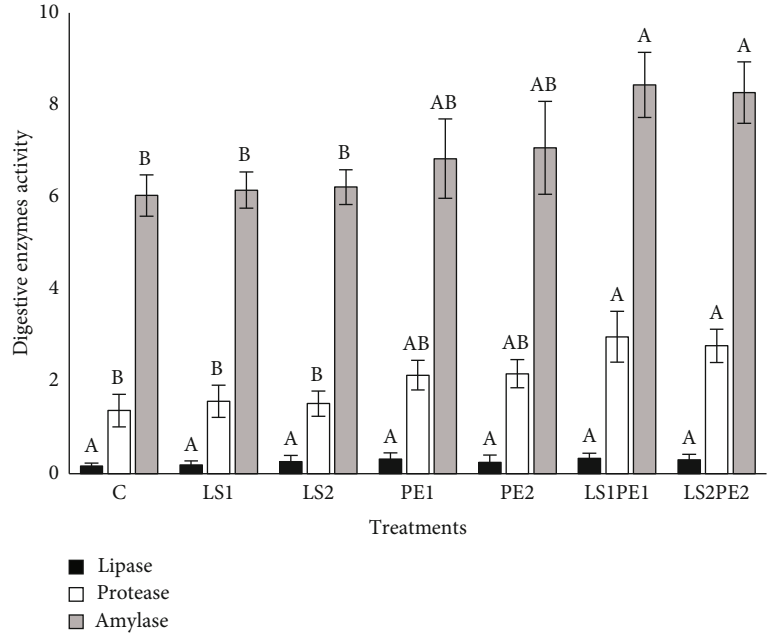

Figure 1: Digestive enzymes activity including lipase, protease, and amylase narrow-clawed crayfish (*P. leptodactylus*) fed with basal diet and supplemented with LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L. salivarius*, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*, 1×10^7 CFU/g + Pectin, 5 g/kg), and LS2PE2 (*L. salivarius*, 1×10^9 CFU/g + Pectin, 10 g/kg) for 18 weeks. Bars represent the mean ± SD ($n = 3$). Different superscripts show significant differences between the experimental groups.

4. Discussion

In modern aquaculture, there is great attention on developing feed additives to boost immune response and fish health, which in turn leads to improved disease resistance [10, 50]. Dietary interventions with functional probiotics/prebiotics could exert beneficial effects on the host through influencing nutrition, gut bacterial ecosystem, metabolism, and immune function [50–52]. In the current study, growth performance and FCR in animals fed with LS or PE were beneficially altered at the end of the feeding trial. However, the highest growth indices and the lowest FCR were obtained in specimens treated by LS1PE1. Therefore, synbiotics had a greater impact than probiotics or prebiotics on growth performance and nutritional efficiency. In agreement with our results, the simultaneous employ of lactobacillus and prebiotics such as pectin + *L. plantarum* [30], oyster mushroom + *L. plantarum* [53], galactooligosaccharide + *L. plantarum* [54] in *L. vannamei* elevated growth indices and feed utilization in *L. vannamei*. These protective effects on host growth can be linked with releasing endogenous enzyme, producing metabolites from the fermentation process (vitamins, SCFs), and improving gut epithelial cells, which in turn facilitate the digestibility and absorption of nutrients [30, 40]. Other studies proposed that the beneficial effects of synbiotics are related to the ability of prebiotics in enhancing the

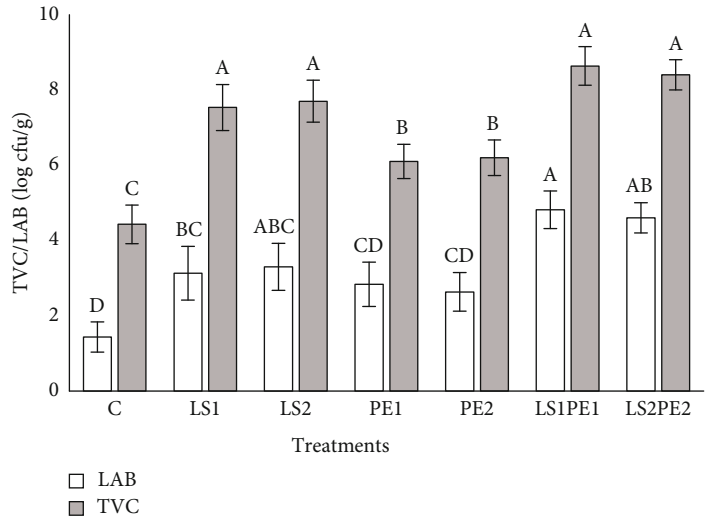

Figure 2: The TVC (total viable heterotrophic bacteria count) and LAB (lactic acid bacteria) of crayfish narrow-clawed crayfish (*P. leptodactylus*) fed with basal diet and supplemented with LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L. salivarius*, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*, 1×10^7 CFU/g + Pectin, 5 g/kg) and LS2PE2 (*L. salivarius*, 1×10^9 CFU/g + Pectin, 10 g/kg) for 18 weeks. Bars represent the mean ± SD ($n = 3$). Different superscripts show significant differences between the experimental groups.

Table 3: Hemolymph indices of narrow-clawed crayfish (*Postantacus leptodactylus*) fed with basal diet and supplemented with LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L. salivarius*, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*, 1×10^7 CFU/g + Pectin, 5 g/kg) and LS2PE2 (*L. salivarius*, 1×10^9 CFU/g + Pectin, 10 g/kg) for 18 weeks.

Parameters	Treatments						
	Control	LS1	LS2	PE1	PE2	LS1PE1	LS2PE2
THC ($\times 10^5$ cell mL^{-1})	125.50 ± 3.50^{d}	149.16 ± 1.90^{bc}	148.60 ± 5.28^{bc}	143.36 ± 2.51^{c}	142.10 ± 2.74^{c}	159.96 ± 2.93^{a}	155.86 ± 3.09^{ab}
HC ($\times 10^5$ cell mL^{-1})	70.83 ± 3.32^{b}	81.33 ± 1.52^{a}	82.83 ± 3.40^{a}	83.50 ± 2.78^{a}	84.40 ± 1.90^{a}	86.96 ± 2.87^{a}	85.40 ± 2.42^{a}
SGC ($\times 10^5$ cell mL^{-1})	26.16 ± 1.25^{c}	32.40 ± 1.11^{a}	31.43 ± 0.86^{ab}	28.36 ± 1.28^{bc}	27.20 ± 1.11^{c}	34.50 ± 1.80^{a}	33.30 ± 2.02^{a}
LGC ($\times 10^5$ cell mL^{-1})	28.50 ± 1.50^{e}	35.43 ± 0.62^{abc}	34.33 ± 1.25^{bcd}	31.50 ± 1.51^{cde}	30.50 ± 1.32^{de}	38.50 ± 1.80^{a}	37.16 ± 1.75^{ab}

THC: total haemocyte count; LGC: large-granular count; SGC: semigranular count; HC: hyaline count. Values are presented as mean ± SD. Different superscripts within a row indicate significant differences at $P < 0.05$ ($n = 3$).

Table 4: Nonspecific immunity responses of narrow-clawed crayfish (*Postantacus leptodactylus*) fed with basal diet and supplemented with LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L. salivarius*, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*, 1×10^7 CFU/g + pectin, 5 g/kg) and LS2PE2 (*L. salivarius*, 1×10^9 CFU/g + pectin, 10 g/kg) for 18 weeks.

Parameter	Treatments						
	Control	LS1	LS2	PE1	PE2	LS1PE1	LS2PE2
LYZ (U mL^{-1})	12.51 ± 1.30^{b}	15.14 ± 1.50^{ab}	15.06 ± 1.43^{ab}	14.75 ± 1.31^{ab}	14.45 ± 1.42^{ab}	17.26 ± 2.30^{a}	16.80 ± 1.70^{ab}
PO (U mL^{-1})	1.07 ± 0.10^{b}	1.35 ± 0.15^{ab}	1.29 ± 0.16^{ab}	1.27 ± 0.12^{ab}	1.18 ± 0.13^{ab}	1.58 ± 0.28^{a}	1.41 ± 0.17^{ab}
NOS (U mL^{-1})	10.06 ± 2.00^{c}	14.16 ± 1.25^{bc}	13.86 ± 1.10^{bc}	18.60 ± 1.42^{a}	17.96 ± 1.30^{ab}	16.93 ± 1.90^{ab}	16.43 ± 1.60^{ab}
AKP (U/L)	15.03 ± 1.55^{c}	16.93 ± 1.90^{ab}	17.30 ± 1.70^{ab}	19.26 ± 1.02^{ab}	18.93 ± 2.18^{ab}	21.93 ± 1.90^{a}	21.76 ± 2.25^{a}
ACP (U/L)	9.16 ± 1.25^{a}	9.06 ± 0.90^{a}	9.56 ± 1.15^{a}	10.10 ± 1.35^{a}	10.63 ± 1.82^{a}	11.33 ± 1.25^{a}	11.76 ± 1.12^{a}

LYZ: lysozyme; PO: phenol-oxidase; NOS: nitric oxide synthase; AKP: alkaline phosphatase; ACP: acid phosphatase. Values are presented as mean ± SD. Different superscripts within a row indicate significant differences at $P < 0.05$ ($n = 3$).

colonization of favorable bacteria [55, 56]. During the recent decade, several researchers have confirmed the role of synbiotics on microbiota in crustaceans. For example, Safari et al. [7] and Safari and Paolucci [8] indicated the modulating effects of *Enterococcus faecalis* + xylooligosaccharide, *Pediococcus acidilactici* + mannanoligosaccharide, and E. *faecalis* + galactooligosaccharide on the gut microbiota of crayfish. These results are consistent with the findings of our study that in the presence of pectin, the rate of LAB colonization increased in the gut. The study implied that LS could use from pectin as a carbon source during the fermentation process, a hypothesis previously confirmed by Kuo

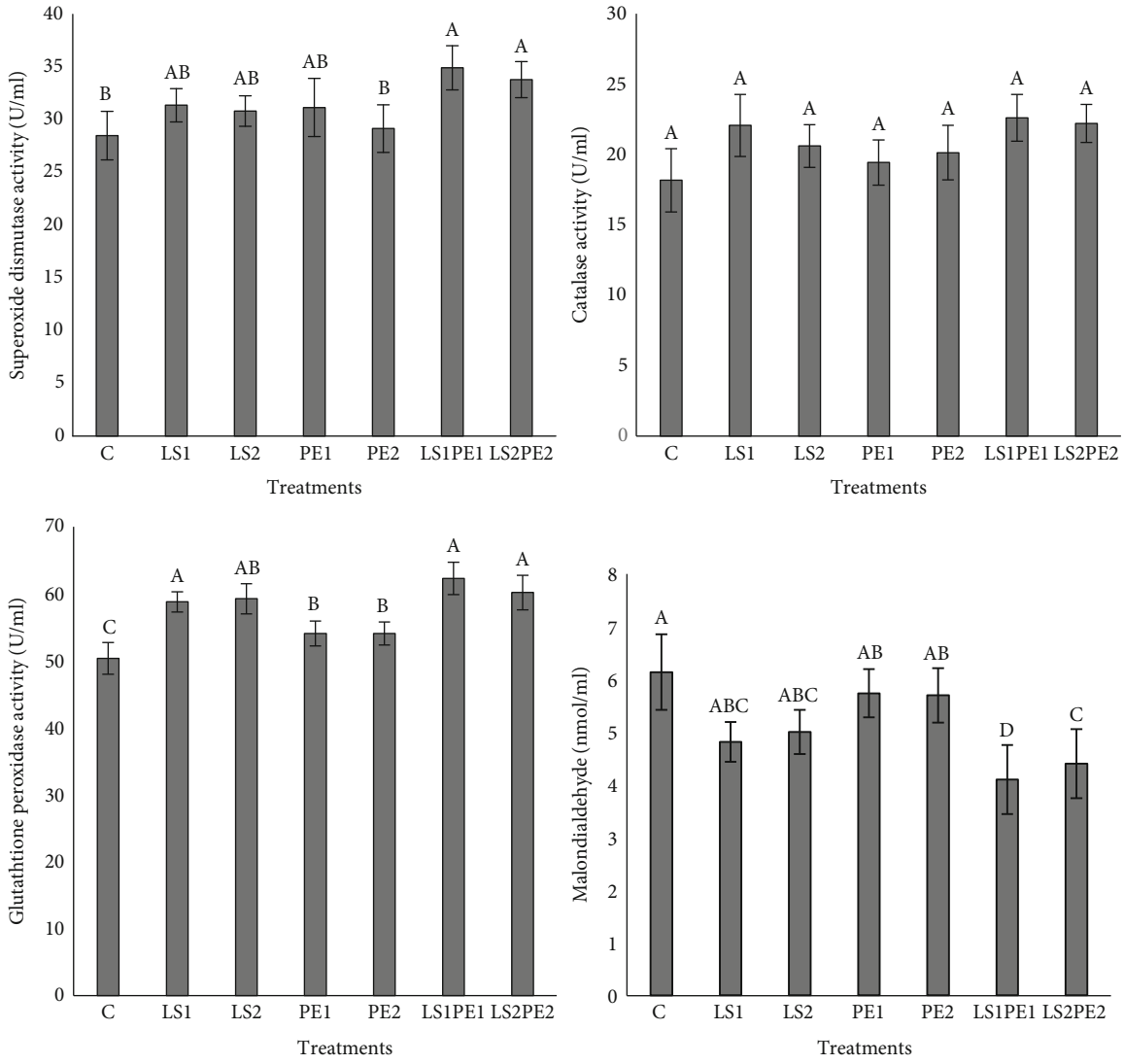

FIGURE 3: Antioxidant enzymes activities of crayfish narrow-clawed crayfish (*P. leptodactylus*) fed with basal diet and supplemented with LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L. salivarius*, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*, 1×10^7 CFU/g + Pectin, 5 g/kg), and LS2PE2 (*L. salivarius*, 1×10^9 CFU/g + Pectin, 10 g/kg) for 18 weeks. Bars represent the mean ± SD ($n = 3$). Different superscripts show significant differences between the experimental groups.

et al. [30] in *vitro* and in *vivo* studies. On the other hand, the successful colonization of LAB can be an inducing factor for the secretion of intracellular and extracellular digestive enzymes [57]. In the current study, a notable improvement in amylase and protease enzymes activities was recorded in narrow-clawed crayfish fed LS1PE1 and LS2PE2 diets. Several works also have confirmed elevated digestive enzymes activities in crustaceans such as *L. vannamei* [58, 59] and narrow-clawed crayfish [8] after feeding by synbiotic. However, more works are needed to elucidate the mechanism of PE + LS on the level of digestive enzymes synthesized by LAB or the host.

Despite the lack of a specific immune system in crustaceans, these animals benefit from two efficient cell-mediated mechanisms to trigger a nonspecific immune system and combat with invader agents. (1) A set of cellular responses are executed directly by immune cells, such as phagocytosis, nodule formation, and encapsulation of pathogens. (2) Humoral molecules secreted by the immune cells including antimicrobial peptides, proteins involved in hemolymph coagulation, and the prophenoloxidase system (proPO) [60]. These two mechanisms are associated with three types of haemocytes, including HCs, GCs, and SGCs [61]. It is generally accepted that the number of circulating haemocytes is a valid marker for assessing the health status of crustaceans [62]. In the current study, the dietary supplementation of LS or PE significantly increased THC and HC. Besides, LGC and SGC count are notable elevated in LS1, LS2 and LS1PE1, and LS2PE2. Similarly, the hemolymph indices were increased in *A. leptodactylus* fed with the *Lactobacillus plantarum* diet at 10^7 CFU/g [6]. Another study on *A. leptodactylus* also indicated that feed incorporated with

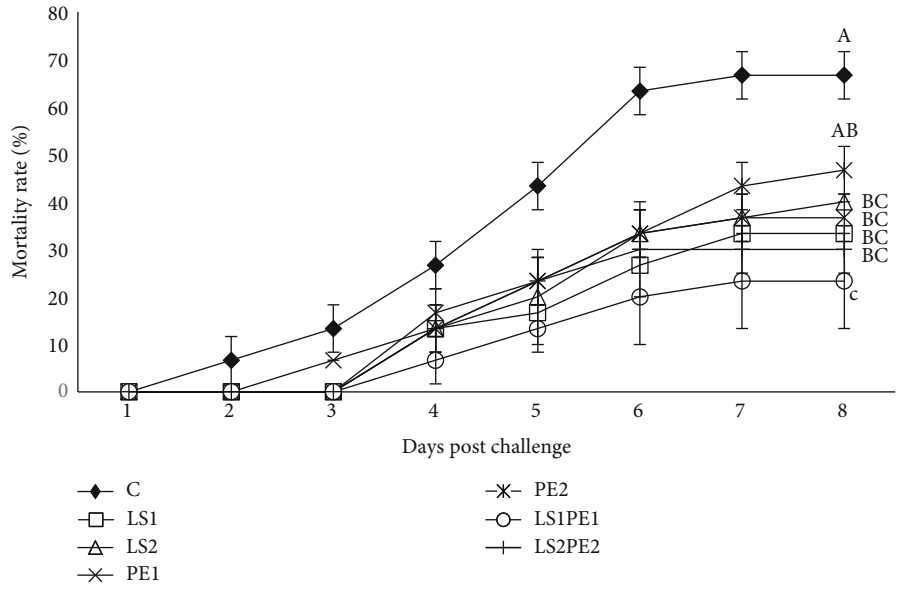

Figure 4: Mortality rate of crayfish narrow-clawed crayfish (*P. leptodactylus*) fed with basal diet and supplemented with LS1 (*L. salivarius*, 1×10^7 CFU/g), LS2 (*L. salivarius*, 1×10^9 CFU/g), PE1 (Pectin, 5 g/kg), and PE2 (Pectin, 10 g/kg), LS1PE1 (*L. salivarius*, 1×10^7 CFU/g + Pectin. 5 g/kg) and LS2PE2 (*L. salivarius*, 1×10^9 CFU/g + Pectin, 10 g/kg) for 18 weeks. Bars represent the mean ± SD ($n = 3$). Different superscripts show significant differences between the experimental groups.

synbiotic (*Enterococcus faecalis* 7.86 log CFU/g +10 g/kg xylooligosaccharide) remarkably increased THC, HC, LGC, and SGC counts [7]. Enhancing the circulating haemocyte count through diet manipulation could induce the activity of humoral factors. Phenoloxidase is the final product in the prophenoloxidase (proPO) system and its activation plays a vital role in the recognition and control of hemolymph infection [63]. Lysozyme acts as an antibacterial molecule in the hydrolysis of the bacterial peptidoglycan layer. Our findings revealed that supplementation of LS1PE1 elevated the PO and LYZ levels of narrow-clawed crayfish serum [64]. These results are in agreement with the immunity findings of *L. vannamei* after feeding with pectin and probiotics, synergistic effects between pectin and *L. plantarum* were also observed [30]. Alkaline phosphatase (AKP) is a type of lysosomal enzyme related to various key functions such as hydrolytic activities, antibacterial, and wound healing in all animals [65]. Acid phosphatase (ACP) is another known lysosomal enzyme in the degradation of microbial pathogens and is considered an indicator to assess the capability of macrophages to digest invader agents [63]. Crayfish fed with diets containing LS, PE, and LSPE illustrated a significant increase in AKP activity in serum when compared to control. Similarly, mannanoligosaccharide + *Pediococcus acidilactici* and xylooligosaccharide + *E. faecalis*, improved the activity of AKP in the hemolymph of crayfish [7]. Our findings demonstrated that LS or PE added to the crayfish diet beneficially regulated AKP activity in crayfish. Nitric oxide (NO) is a bactericidal molecule against extracellular and intracellular pathogens in the nonspecific immune systems of many organisms [66]. In the current study, NOs activity was remarkably influenced by diets probiotic (LS, LSPE). Similarly, a rise in NOs level was reported using dietary *E. faecalis* + galactooligosaccharide and *E. faecalis* + mannanoligosaccharidein in *A. leptodactylus* [8]. These results confirmed that the compounds generated in the presence of PE by LS can be useful for regulating the immunocompetence of crayfish via both the complementary and synergistic effects. Enhancing disease resistance against common pathogens is the end product of dietary supplements. In the current study, diets containing synbiotic (pectin + probiotic) elevated the survival rate of the narrow-clawed crayfish infected with *A. hydrophila*. Indeed, LS1PE1 administrated into diet protected narrow-clawed crayfish up to 44% against infection. The finding is consistent with the observers of other authors, who indicated the stimulating effects of synbiotics on the immunocompetence and resistance of shellfish and finfish species against biological stressors [56, 67, 68]. Increased colonization of lactic acid bacteria in the presence of pectin, the release of bacteriocins, and boosting of the immune defense elements including LYZ, AKP, NO, PO, and haemocytes are possible mechanisms to enhance disease resistance in LSPE groups.

Coping with oxidative stress in aquatic animals is accomplished through a set of antioxidant enzymes such as SOD, CAT, and GPx [69]. These enzymes play a vital role in maintaining cell integrity by scavenging reactive oxygen species (ROS) [70]. However, in harsh conditions, the host's antioxidant capacity is not adequate to scavenge ROS, thus external resources are needed to boost antioxidant capacity [71, 72]. Adding stimulant compounds to the aquafeed is one of the commonest ways to improve the antioxidant defense in fish. In this study, the dietary LSPE indicated high antioxidant capacity where SOD and GPx activities remarkably increased. Besides, MDA levels as a good marker for

measuring oxidative stress (imbalance between antioxidant defense and ROS level) were significantly reduced in response to synbiotic diets [70]. These results may be because of the ability of pectin to boost antioxidant capacity or ROS scavenging, previously reported in carp by Hoseinifar et al. [73]. On the other hand, the beneficiary properties of probiotics on the antioxidant capacity may be associated to resist ROS, chelating ions, and producing compounds with antioxidant properties such as folate, butyrate, and glutathione [74]. Therefore, inclusion synbiotic in the narrow-clawed crayfish diet can enhance the antioxidant power of the crayfish and support the cells versus the harmful effects of oxidative stress.

5. Conclusion

In conclusion, the present findings indicated that the dietary *L. salivarius* or pectin could enhance growth performance and hemolymph indices. However, administration of synbiotic, especially LS1PE1, had better performance on digestive enzyme activity, modulation of gut microflora, nonspecific immune responses, antioxidant capacity, as well as disease resistance against *Aeromonas hydrophila*. Accordingly, dietary supplementation with LS1PE1 can be considered a beneficial feed additive in crayfish diet.

Data Availability

The data are available from the corresponding authors upon reasonable request.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Samyah Darwish Saddig Jastaniah worked on the conceptualization. Hafsan Hafsan was assigned to data curation. Cheng-jui Tseng was responsible for the formal analysis. Yasir Salam Karim was responsible for the funding acquisition. Mohammed Ubaid Hamza worked on the investigation. Mahnaz Dadras and Mohammad Mansouri Chorehi constructed the methodology, field study, and sampling. Noora M. Hameed was assigned in project administration. Sura Hasan Al-Zubaidi worked on the resources. Saif Sabbar Kemil Almotlaq supervised the study. Ghulam Yasin was assigned in the visualization. A. Heri Iswanto was responsible in writing–original draft. Samyah Darwish Saddig Jastaniah, Cheng-jui Tseng, Mahnaz Dadras, and Saif Sabbar Kemil Almotlaq were responsible in writing, reviewing, and editing the manuscript.

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