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Effects of Dietary *Lactobacillus plantarum* on Growth Performance, Digestive Enzymes and Gut Morphology of *Litopenaeus vannamei*

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Abstract A 15-day feeding trial was conducted to investigate the effect of dietary *Lactobacillus plantarum* on growth performance, digestive enzyme activities and gut morphology of juvenile Pacific white shrimp, *Litopenaeus vannamei* (initial body weight = 7.96 ± 0.59 g). Four microbound diets were formulated to contain fermentation supernatant (FS), live bacteria (LB), dead bacteria (DB), and cell-free extract (CE) of *L. plantarum*. Results indicated that final weight was significantly higher in FS, DB, and CE group in comparison to the control group ($P < 0.05$). The maximum weight gain rate (WGR) and specific growth rate (SGR) of the CE diet group were significantly higher than that of other groups ($P < 0.05$). The FCR of CE diet group was lower than that of the control, LB, DB, and FS diets groups ($P < 0.05$). The highest digestive enzyme activities (amylase, lipase, and pepsin activity) in the hepatopancreas and gut of shrimp were observed in the CE diet group. Histological study revealed that dietary CE diet could significantly increase the enterocytes height of shrimp. The administration of cell-free extract of *L. plantarum* could effectively improve the growth performance of *L. vannamei* via the improvement of digestive enzyme activities and the enterocytes height of shrimp. The results of this study will be essential to promote application of probiotics in shrimp aquaculture.

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Keywords *Lactobacillus plantarum* · *Litopenaeus vannamei* · Growth performance · Digestive enzymes · Gut morphology

Introduction

Due to its tolerance to wide range of environmental conditions, fast growth and high economic value, Pacific white shrimp, *Litopenaeus vannamei*, has been considered as one of the most important mariculture shrimp species around the world [1]. Food and Agriculture Organization (FAO) has shown a huge increase in Pacific white shrimp production from 154,515 t in 2000 to 3,668,682 t in 2014, registering an increase of nearly 2274%. Feed cost is one of the major spending in shrimp culture, which typically account for more than 70% of the production cost. The problem of low feed utilization in shrimp culture has caused the serious economic loss throughout the world [2]. Therefore, enhancing the growth performance of shrimp and improving feed utilization are critical goal of aquaculture industries and scientific researchers. Several researches approved that application of probiotics has become an ecofriendly health management strategy to improve growth performance, feed utilization, and digestibility of dietary ingredients of shrimp in aquaculture [3–7].

Lactobacillus plantarum is a rod-shaped, gram positive, catalase negative, and non-spore forming facultative anaerobic bacterium, which belongs to the lactic acid bacterium (LAB), and has been widely used as a live diet supplement. *L. plantarum*, when used as a diet supplement, colonize the gut of host and improve feed utilization by the synthesis of growth factors such as vitamins, cofactors, fatty acids, and amino acids and can also augment digestive enzymes activity of target animal which increases nutrient absorption and

growth of the host. Previous studies have demonstrated that *L. plantarum* could improve the growth performance and feed efficiency in *Macrobrachium rosenbergii* [8] and *L. vannamei* [3], increase digestive enzyme activities in *Portunus pelagicus* [9], and improve immunity, disease resistance, and survival in *L. vannamei* [3] and *Marsupenaeus japonicas* [10]. In addition, there is some information regarding its increasing growth performance efficacy as a heat-killed diet supplement in teleost models viz. Red seabream *Pagrus major* [11, 12], Amberjack *Seriola dumerili* [13] as well as in crustacean models viz. *M. rosenbergii* [14] and *M. japonicas* [15].

Although the probiotic potential of live or heat-killed *L. plantarum* used as a diets supplement in shrimp has been elucidated, the effective ingredient and exact mechanism of which have not been reported. Therefore, this study aimed to investigate the effects of *L. plantarum* in different treatment supplementation on the growth performance, digestive enzyme activity, and gut morphology of *L. vannamei*. The results of the study will be essential to understand the roles of *L. plantarum* in health management of shrimp aquaculture.

Materials and Methods

Shrimps and Bacterial Strains

Healthy juvenile shrimp were collected from a local hatchery and reared in a semi-intensive culture pond at Shenzhen Base, South China Sea Fisheries Research Institute of Chinese Academy of Fishery Sciences (Shenzhen, China). They were acclimatized in an aerated seawater tank at room temperature (25–27 °C), and fed with a commercial diet four times (6:00, 11:30, 17:30, and 23:30) per day for 1 week under controlled environment prior to this study.

The probiotic used in this study was a commercial *L. plantarum* (provided by Xinhailisheng Biological Technology Co., Ltd., South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, China) containing cells with a count of 10^9 CFU mL^{-1}. *L. plantarum* isolated from the gut contents of the tropical freshwater fish *Oreochromis mossambicus* was used in this study. Bacteria were cultured in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) for 24 h at 37 °C. Cell density was calculated from OD_{600} values and correlated with colony forming unit (CFU) counts using serial dilution and spread plating on MRS agar. The quantified bacteria were maintained at 4 °C in a suspended form and were used for feed preparation as required.

Experimental Diet

A pretreated commercial manufactured feed for shrimp (Evergreen Feed Co. Ltd., China) was used as a dietary source in this study. The ingredients of the commercial feed were crude protein 40%, crude fiber 5%, crude ash 15%, crude fat 6%, water 12.5%, and so on. For probiotic treatment group, four experimental diets were prepared [16] as follows:

Fermentation supernatant group (FS): fresh fermentation supernatant was collected after centrifuging the bacterium solution at 4000×*g* for 20 min.
Live bacteria group (LB): bacterial cells were harvested by centrifugation, washed twice with sterile saline, adjusted to obtain 10^9 CFU ml^{-1} cell slurry.
Dead bacteria group (DB): cell slurry was heated in water bath (65 °C, 30 min).
Cell-free extract group (CE): cell slurry was broken by Sonifier cell disrupter (2 k Hz, 40 min).

The control diet was sprayed with sterile saline. Each group was tested 3 ml solution and then was sprayed to 300 g of the commercial formulated feed and mixed. The supplemented feed packed in tight container was stored at 4 °C, and used up within 2 days. The bacterial count of the diet was examined every week using the spread plate method to verify the concentration of the probiotic, and to check for possible contamination.

Experimental Design and Daily Management of Shrimp

Fifteen indoor fiberglass tanks (800 L) were used for the feeding experiment and there were 100 shrimps (initial body weight = 7.96 ± 0.59 g) in each tank. The tanks containing the shrimps were divided into five treatment groups with three replicates. Shrimp of the treatment groups were fed with the four diets supplemented with fermentation supernatant (FS), live bacteria (LB), dead bacteria (DB), and cell-free extract (CF) of *L. plantarum*, respectively, whereas control group shrimp were fed with sterile saline diet at 3–5% body weight for 15 days. The shrimp were fed 4 times per day at 6:00, 11:30, 17:30, and 23:30. During the feeding trial, the amount of diet given was progressively adjusted according to the feed consumption of the shrimp by checking the remaining excess feed at the bottom of the tanks after feeding for 1 h. Thus, overfeeding was minimized and shrimp were fed close to satiation. Every morning and afternoon before each feeding time, all tanks were cleaned by siphoning off accumulated uneaten feed, feces, molts, and dead shrimp. Uneaten feed particles were dried, weighed, and used for correction of feed intake.

The tanks were equipped with continuous aeration and maintained under natural light/dark regime. Water quality including salinity (30–32 ppt), temperature (23–27 °C), pH (7.8–8.2), dissolved oxygen (6.8–7.0 mg L^{-1}), and ammonia nitrogen (0.3–0.4 mg L^{-1}) were controlled daily.

Growth Parameters

At the end of the experiment, 10 shrimp were randomly sampled from each tank for the growth performance measurement. The weight gain (WGR), feed conversion ratio (FCR), and specific growth rate (SGR) were determined using the following equations:

$$\text{WGR}\ (\%) = 100 \times (W_t - W_o)/W_o$$

$$\text{FCR}\ (\%) = \textit{Total feed given}/(W_t - W_o)$$

$$\text{SGR}\ \left(\%\text{day}^{-1}\right) = 100 \times [\log W_t - \log W_o]/t$$

Where, W_t is the weight of shrimp in sample time and W_o is the initial average weight of shrimp, while log W_t is the logarithm of final average weight of shrimp and log W_o is the logarithm of initial average weight of shrimp and "t" represents the days of culture.

Digestive Enzyme Analysis

The whole of hepatopancreas and gut of four shrimp from each tank were randomly sampled, and homogenized by adding sterile 0.9% saline solution to prepare 10% (W:V) homogenates. Homogenates were centrifuged at 5000 *g* for 20 min at 4 °C. After removing precipitates, supernatants were immediately kept at −80 °C for the digestive enzyme activity analyses. The activities of amylase, lipase, and pepsin were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions with a spectrophotometer (xMark, Bio-Rad, USA). Tissue protein contents in crude extracts were determined with the Coomassie Brilliant Blue protein assay kit (Jiancheng, Ltd., Nanjing, China).

Amylase catalyzes the hydrolysis of starch and the unreacted starch can react with iodine solution to generate blue complex at 660 nm. One amylase activity unit is defined as 10 mg starch hydrolyzed within 30 min at 37 °C with enzymes in 1 mg protein.

Lipase activity was determined through measuring the free fatty acids production from enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil at 420 nm. One lipase activity unit is defined as 1 g tissue protein reacts with 1 μmol substrate in the reaction system at 37 °C.

Pepsin catalyzes the hydrolysis of protein and the product of Tyrosine can reduce the phenol reagent to blue compound at 660 nm. One pepsin activity unit is defined as 1 μg Tyrosine generated with protein hydrolysis catalyzed by enzymes within 1 mg protein.

Intestinal Histological Examination

The midgut of three shrimp from each tank was randomly sampled for histological examination. The mid gut was immersion fixed in Bouin's solution for 18 h and then transferred to 70% (*v*/*v*) ethanol until processing [17]. Sections (5 μm) were made using a rotary microtome, stained with hematoxylin and eosin, and examined under a light microscope (×40 magnification). The height of the intestinal epithelial cells was quantified within five randomly selected visual fields from triplicates in each treatment. The intestinal epithelial cells were chosen since these cells are responsible for nutrients absorption.

Statistical Analysis

The data were presented as the mean ± SEM. Statistical analyses were conducted using SPSS software (Ver 22.0), and determined using one-way ANOVA and post hoc Duncan multiple range tests. Significance was set at $P < 0.05$.

Result

Growth Parameters

The growth parameters of shrimp in different treatments were shown in Table 1. The final weight of shrimp in FS, DB, and CE treatment was significantly higher than the control ($P < 0.05$) after 15 days. The maximum WGR and SGR were observed in shrimp fed CE diet, followed by shrimp fed DB, FS, and LB diets, and finally the control diet ($P < 0.05$). FCR of shrimp fed CE diet was lower than that of shrimp fed the control, LB, DB, and FS diets ($P < 0.05$).

Digestive Enzymes Activity

Amylase, lipase, and pepsin activity in hepatopancreas of LB, DB, CE, and FS diet group increased significantly compared to the control group ($P < 0.05$, Fig. 1a). Amylase, lipase, and pepsin activity in hepatopancreas of CE group were the highest (2.04, 2.63, 3.10-fold of the control group, respectively, $P < 0.05$).

The maximum amylase activity in gut was observed in CE diet group, which was significantly different from that of other diet groups ($P < 0.05$). Compared to the control group, the lipase and pepsin activity in gut of CE and FS diet groups were significantly higher ($P < 0.05$, Fig. 1b).

Gut Morphology

The results of the histological examination of enterocytes morphology were presented in Fig. 2. Observations at the

Table 1 Effect of five experimental diets on the growth performance of *L. vannamei* ($N = 3$)

Treatments	CG	LB	DB	CE	FS
IBW (g)	8.10 ± 0.13^{a}	7.87 ± 0.18^{a}	8.00 ± 0.12^{a}	7.71 ± 0.12^{a}	8.10 ± 0.20^{a}
FBW (g)	12.02 ± 0.27^{a}	12.60 ± 0.26^{ab}	13.03 ± 0.25^{b}	15.27 ± 0.34^{c}	13.00 ± 0.26^{b}
WGR (%)	48.31 ± 2.84^{a}	60.14 ± 2.61^{b}	62.81 ± 3.40^{b}	98.12 ± 3.52^{c}	60.43 ± 1.93^{b}
FCR (%)	1.21 ± 0.07^{c}	0.98 ± 0.04^{b}	1.00 ± 0.06^{b}	0.60 ± 0.02^{a}	1.00 ± 0.03^{b}
SGR (%)	1.14 ± 0.06^{a}	1.36 ± 0.05^{b}	1.41 ± 0.06^{b}	1.98 ± 0.05^{c}	1.37 ± 0.03^{b}

Values (mean ± SEM) with *different superscript* in a row show significant differences ($P < 0.05$)

intestinal mucosa demonstrated that the maximum enterocytes height was observed with shrimp fed CE diet ($P < 0.05$). Histological study revealed that compared with the control group, the apical brush border in *L. plantarum* groups appeared normal, maintained by the presence of intercellular tight junctions, which span the gap from cell to adjacent cell, with no signs of necrotic enterocytes or cell damage observed (Fig. 3).

Discussion

Ever since the use of *L. plantarum* in aquaculture, a growing number of studies have demonstrated that live or dead *L. plantarum* were effective as dietary supplement and immunostimulant in fish and shrimp [12, 18–21]. However, there is limited information regarding its efficacy as fermentation supernatant (FS) or cell-free extract (CE) diet supplement in *L. vannamei*. In addition, there is no study comparing the effects of four diets supplement, such as fermentation supernatant (FS), live bacteria (LB), dead bacteria (DB) and cell-free extract (CE) of *L. plantarum* on growth performance of white shrimp.

In the present study, the final weight, WGR, FCR, and SGR have significantly improved in all treatment groups (LB, DB, CE, and FS) compared to the control (Table 1). Although several studies have demonstrated the beneficial effects of probiotics on the growth performance in shrimp [6, 22, 23], the exact mechanism of action is not well understood by now. The first explanation could be related to the induction of digestive enzymes, including amylase, lipase, and pepsin, which consequently stimulate the natural digestive enzyme activities of the host [24, 25]. *Lactobacilli* in aquaculture organisms can produce a range of digestive enzymes such as protease and amylase [26]. It did not distinguish the activity due to enzymes synthesized by the shrimp or the probiotics. However, the exogenous enzymes produced by the *L. plantarum* would show a small contribution to the digestive enzyme activities of the shrimp in our experiment. In present study, the higher level of total digestive enzyme activity was recorded in shrimp fed LB, DB, CE, and FS diets where the better growth performances were observed compared to the control (Fig. 1). Similar results have been reported by Zokaeifar et al. [27] who observed a higher digestive enzyme activity in shrimp (*L. vannamei*) treated with *Bacillus subtilis* than the control. Another possible explanation for the improvement of the shrimp growth factors

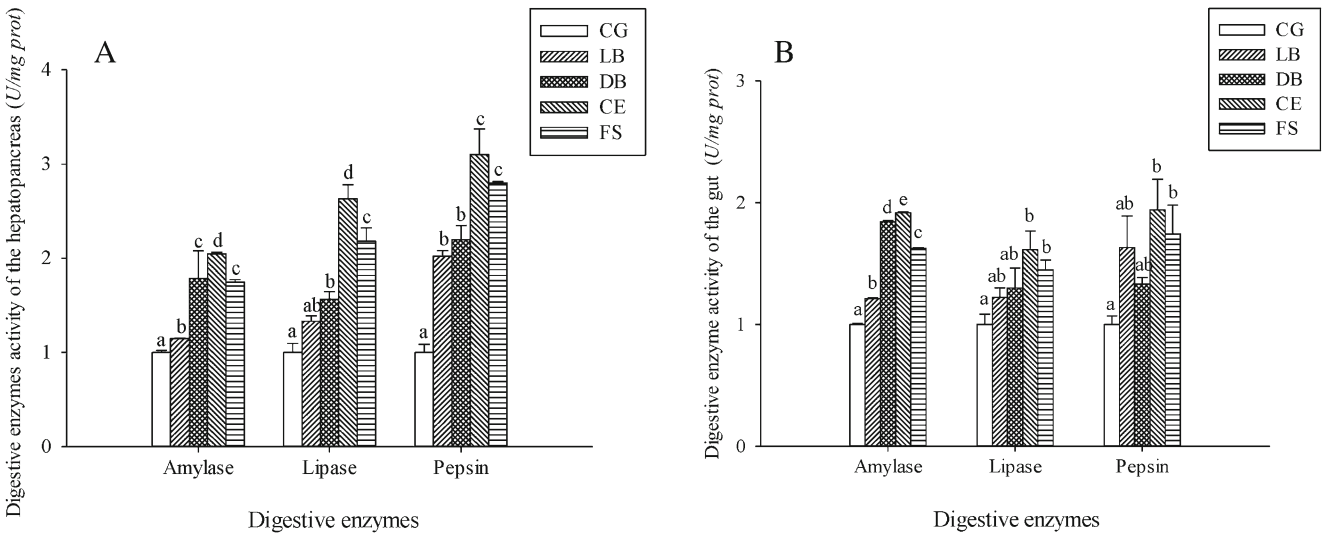

Fig. 1 Digestive enzyme activity of *L. vannamei* in hepatopancreas (**a**) and gut (**b**) in four treatments and control after 15 days trial ($N = 3$). *Vertical bars* represented the mean ± SEM. *Different letters* represent significant differences between treatments

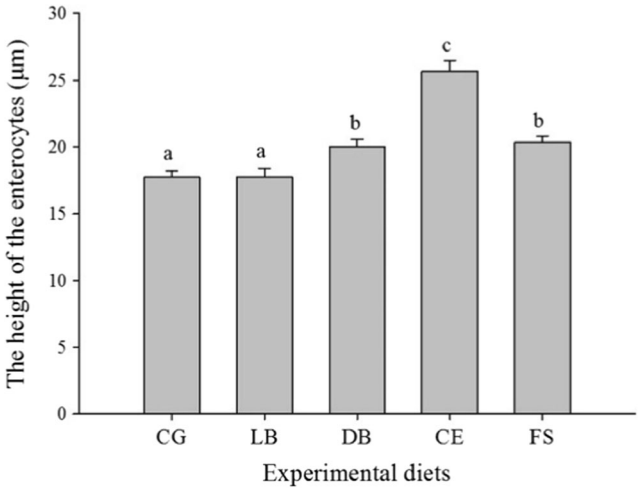

Fig. 2 Intestine morphology of *L. vannamei* fed on five different experimental diets. Data represent the mean ± SEM. *Different letters* represent significant differences between treatments

by four different treatments of *L. plantarum* may be due to the height and density of enterocytes. Intestinal epithelial cell microvilli provide a vast absorptive surface area, the increase in enterocytes height and/or density can increase nutrient absorptive ability [28, 29]. The results of the present study showed that LB, DB, CE, and FS diets could increase the height and density of shrimp enterocytes, which suggested that dietary *L. plantarum* could improve its nutrient absorptive ability.

Mannan oligosaccharide (MOS) is derived from cell wall of *Saccharomyces cerevisiae* and is widely used in nutrition to improve growth performance and enhance gastrointestinal health of aquatic animals [28–30]. In this study, the higher level of total digestive enzyme activity in hepatopancreas and gut, the higher level of height and density of shrimp enterocytes, and the better growth performances were observed in shrimp fed CE diet compared to all other treatment groups and control. These results indicated that administration of dietary cell-free extract of *L. plantarum* supplementation in *L. vannamei* have more significant beneficial affection than the live bacteria, dead bacteria, and fermentation supernatant of *L. plantarum*.

The intestine of shrimp is relatively short therefore, live preparation of *L. plantarum* which has a cell wall was difficult to be digested and can easily excreted in the stool [31]. Compared to the live bacteria of *L. plantarum*, cell-free extract of *L. plantarum* supplementation was easier to be digested by the shrimp thereby had better growth performance. The present results were consistent with the previous studies, where the similar improvement in growth performance has been reported in aquatic animal fed components derived from *Lactobacillus* and yeast [13, 30, 32]. When used as a live diet supplement, *L. plantarum* has been found to inhibit the adhesion and growth of pathogenic bacteria and improves immunity, disease resistance, and survival in host by producing and secreting antibacterial compounds [33, 34]. However, there is no information regarding its efficacy as cell-free extract diet supplement in *L. vannamei* about immune response.

Dash et al. [14] found incorporation of heat-killed *L. plantarum* in diets improved the growth and feed utilization parameters of *M. rosenbergii*. Dawood et al. [12] observed that Rea seabream *P. major* juveniles fed a diet containing heat-killed *L. plantarum* displayed significantly increased

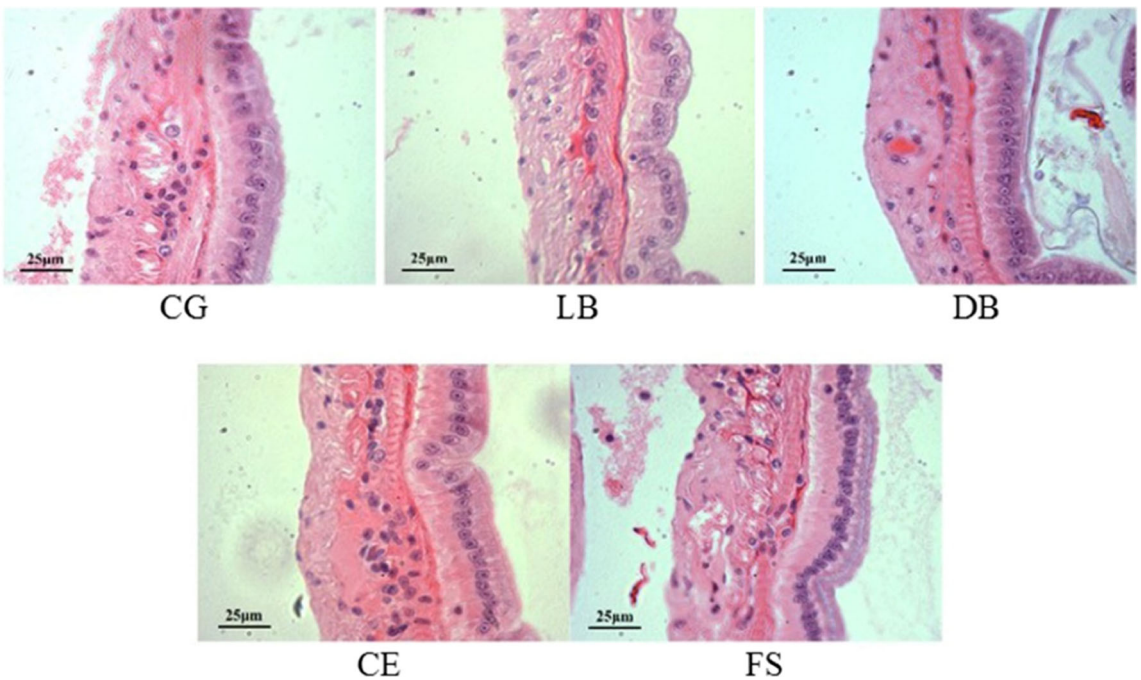

Fig. 3 Light micrographs from the gut region of *L. vannamei* fed on five different experimental diets after the 15 days trial. *Scale bar* = 25 μm

growth performance compared to the control fed group. According to the results of these studies, intake of heat-killed *L. plantarum* enhanced growth performance of aquatic animals. However, there was a thick layer of cell wall outside of heat-killed *L. plantarum* (dead bacteria), which was difficult to be digested by shrimp and its intestinal bacteria. The cell-free extract of *L. plantarum* has low-molecular-weight nutrients and has no thick layer of cell wall covering the bacteria outside thereby more can easily be assimilated by the shrimp and had better growth performance.

Compared to the fermentation supernatant of *L. plantarum*, cell-free extract supplementation has more nutritional components, such as single-cell protein (SCP) [35], cell surface-associated lipoteichoic acid (LTA) [36], or surface-layer proteins (Slps) [37]. Shrimp and intestinal bacteria of shrimp can digest these nourishing substances more easily which led to improved nutrient digestion and feed utilization. In the present study, histological examination showed that dietary CE could significantly increase the height of enterocytes of *L. vannamei*, which suggested that dietary simple and absorbable low-molecular-weight metabolites were assimilated by the intestine, could improve its nutrient absorptive ability, and had better growth performance.

In conclusion, the results indicated that fermentation supernatant of *L. plantarum*, live bacteria, dead bacteria, and cell-free extract supplementation could be used to improve the growth performance, gut morphology, and digestive enzymes of *L. vannamei*. Considering the effect on growth performance and economic benefits, the best supplementation in the diet should be cell-free extract of *L. plantarum*. Further research needs to be conducted to identify the mechanisms of the fermentation supernatant of *L. plantarum*, live bacteria, dead bacteria, and cell-free extract supplementation action on stress resistance, immune response, and gut microbiota of shrimp.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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