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Effect of *Clostridium butyricum* in different forms on growth performance, disease resistance, expression of genes involved in immune responses and mTOR signaling pathway of *Litopenaeus vannamai*

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ABSTRACT

A 42-day feeding trial was conducted to evaluate the effects of diet supplemented with various additives from *Clostridium butyricum* (fermentation supernatant, FS; live cells, LC; cell-free extract, CE; spray-dried spores, DS; mixture of live cells and supernatant, LCS) on the growth, intestinal morphology, disease resistance, immune gene expression and mTOR signaling-related gene expression in *Litopenaeus vannamai*. The feeding trial showed that the final weight and specific growth rate of the shrimp were improved significantly while the feed conversion ratio were reduced significantly in LC, CE, DS and LCS groups compared to the control. The villus height and intestinal wall thickness of shrimp's mid-intestine in LC, DS and LCS group increased significantly. After challenge test to *Vibrio parahaemolyticus*, the cumulative mortalities of the shrimp in LC, CE, DS and LCS groups were significantly lower than that of the control. As compared to the control, the relative expression levels of superoxide dismutase, lysozyme, prophenoloxidase (proPO), Toll, Immune deficiency (Imd), Relish, TOR, 4E-BP, eIF4E1α and eIF4E2 genes in the shrimp of DS and LCS groups enhanced significantly, whereas the relative expression levels of proPO, SOD, Toll, Imd, Relish, elF4E1α and elF4E2 genes were statistically the same between FS group and the control. These results suggested that the spray-dried spores and mixture of live cells and supernatant of *C. butyricum* exerted better probiotic benefits in modulating immune responses of shrimp. In addition, single supernatant could not be helpful to shrimp while mixture of live cells and supernatant could better improve the immune responses of shrimp in comparison to single live cells. The integration of *C. butyricum* and their metabolites supplemented into feed could significantly improve growth performance, intestinal morphology, immunity capacity and resistance against *V. parahaemolyticus* of *L. vannamei*.

1. Introduction

Litopenaeus vannamei has been main object of aquaculture industry in China because it has many advantages, such as strong stress tolerance, strong disease resistance, fast growth and short growth cycle, etc. [1]. Although the production of *Litopenaeus vannamei* is largest in China compared with other countries [1], the increasing intensity degree and exacerbation of diseases problems and the deteriorating of breeding environment has resulted in large economic losses as the aquaculture continues to expand [2]. Many pathogenic bacteria belonging to the genus *Vibrio* are primary pathogenic bacteria for shrimp. In particular, *Vibrio parahaemolyticus* is a causative agent of acute hapatopancreatic necrosis syndrome, which causes early death in white shrimp. Emergence of this condition has brought tremendous economic losses to the aquaculture industry, including in Asia and Latin America (and especially China), since 2010 [3,4].

Indiscriminate use of chemical additives and veterinary medicines as preventative and curative measures for diseases has resulted in antibiotics resistance among pathogenic bacteria and deteriorating environmental conditions [5]. Several alternative methods have been considered to improve the quality and sustainability of aquaculture production. Of these, probiotics have been shown to play an important roles in aquaculture [6]. Probiotics exert many beneficial effects on aquatic animals, such as interference with pathogenic bacteria by competing with nutrients and adhesion sites, improvement of the barrier function of the epithelial lining, immunomodulation and influences on other bodily organs through the immune system and neurotransmitter production [7]. Increasing numbers of probiotics have been

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applied to shrimp farming in recent years [8], including yeast, *Bacillus*, photosynthetic bacteria and *Enterococcus*. *Clostridium butyricum* is an obligate anaerobic, butyric acid-producing, endospore-forming bacteria, which belongs to human and animal intestinal normal flora [9]. Research from Duan et al. [10] revealed that the spores of *C. butyricum* could improve growth performance, modulate intestine digestive capacity, and enhance intestine immune function of *L. vannamei* against ammonia stress. Our previous study found that live cells of *C. butyricum* in suitable doses could significantly improve the growth performance, immunity capacity, and resistance against *V. parahaemolyticus* of *L. vannamei*, and have a positive effect on the intestinal morphological structure [11]. Although the effects of spores or live cells of *C. butyricum* as diet supplements in shrimp have been elucidated, the effective ingredient and exact mechanism thereof have not been reported. Therefore, this study aims to investigate the effects of *C. butyricum* CBG01 in different treatments (i.e., fermentation supernatant, live cells, cell-free extract, spray-dried spores and mixture of live *C. butyricum* and supernatant) on the growth performance, intestinal morphology, disease resistance, the expression levels of immune genes and mTOR signaling-related genes in *L. vannamei*.

2. Materials and methods

2.1. Experimental strain

C. butyricum strain CBG01 was obtained from the Microbial Culture Collection Center, Lab of Aquaculture Ecology, Ocean University of China. The reinforced medium (glucose 10 g, tryptone 10 g, yeast extract 5 g, beef extract 3 g, $(NH_4)_2SO_4$ 1 g, $MnSO_4$ 0.2 g, KH_2PO_4 4 g and distilled water 1000 mL) was used for culturing *C. butyricum* strain CBG01. The bacteria strain was cultured at 37 °C for 48 h in an anaerobic system (YQX-II, Shanghai CIMO Medical Instrument Manufacturing Co., China). Butyrate concentration in the fermentation broth was 0.06 g/mL, detected by Qingdao F&C Testing and Analysing Co., Ltd (Qingdao, China). Cells were harvested by centrifugation (10,000 g, 4 °C, 20 min) and washed twice with sterile saline. The fermentation supernatants of *C. butyricum* CBG01 were filtered (0.22 μm) and stored at 4 °C. Cell-free extract was achieved by breaking the cell slurry using a sonifier cell disrupter (300 W, 20 min). Spray-dried spores of *C. butyricum* were produced by Qingdao GBW Group.

2.2. Experimental animals

Juvenile L. *vannamei* was purchased from Qingdao Baorong Aquatic Product Technological co., Ltd. (Qingdao, China). Prior to the experiment, the shrimp were cultured in a 400 L tank for 14 d and fed with commercial feed purchased from Yuehai Feed Group (Zhanjiang, China). The components of commercial feed were crude protein (40%), crude ash (16%), crude fiber (5%), crude fat (4%), lysine (2.1%), moisture (12%), total phosphorus (1%), NaCl (3%), methionine (0.7%), calcium (4%). The culture conditions (temperature 25 ± 1 °C, salinity 28–30‰, pH 7.9–8.2 and dissolved oxygen > 5 mg/L) were maintained until completion of the experiment.

2.3. Experimental design and management

After fasting for 24 h, 360 similar-sized individuals (approximately 3.74 g in body weight) were randomly selected and distributed into 30 aquariums (53 × 28 × 34 cm; 12 individuals per aquarium). Five probiotic treatments were designed and prepared, i.e., live cell group (LC), commercial feed supplemented with live *C. butyricum* at the dose of 1×10^{11} cfu/kg feed; spray-dried spores group (DS), commercial feed supplemented with spores of *C. butyricum* at the dose of 1×10^{11} cfu/kg feed; cell-free extract group (CE), commercial feed supplemented with cell-free extract of *C. butyricum*, equivalent to the dose of 1×10^{11} cfu/kg feed; fermentation supernatant group (FS), commercial feed supplemented with fermentation supernatant of *C. butyricum* at the concentration of 120 mL/kg feed; mixture of live cell and supernatant group (LCS), commercial feed supplemented with live *C. butyricum* at the dose of 1×10^{11} cfu/kg feed and fermentation supernatant at the concentration of 120 mL/kg feed. The doses of fermentation supernatant supplemented in feed were designed according to the results of previous study [12], in which shrimp supplemented with 7.2 mg/kg sodium butyrate in feed showed significantly improved growth performance of shrimp. Commercial feed was used as the control.

Shrimp were fed with different diets twice daily at 8:00 a.m. and 5:00 p.m. Daily feed consumption was approximately 5% the shrimp's weight [13]. Uneaten feed and faeces were removed by pipetting after feeding 2 h after feeding. In the 42-day feeding trial, the culture conditions (temperature 25 ± 1 °C, salinity 28–30‰, pH 7.9–8.2 and dissolved oxygen > 5 mg/L) were maintained until completion of the experiment.

2.4. Challenge test

The *V. parahaemolyticus* strain which was isolated from diseased shrimp was provided by Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. At the end of the experiment, 15 shrimp were randomly selected from each group and distributed into 3 aquariums (5 individuals per aquarium). Each shrimp was injected intramuscularly in the third abdominal segment with live *V. parahaemolyticus*. The concentration of *V. parahaemolyticus* was 1×10^6 cells/mL via the pre-experiment. Each shrimp was injected with 25 μL of *V. parahaemolyticus* at the concentration of 1×10^6 cells/mL. During the challenge test, the culture conditions and environment conditions were constant, and dead shrimp were collected and recorded for 14 d to determine mortality.

2.5. Intestinal histological observations and growth performance

At the end of the experiment, 15 shrimp from the control, FS, DS, LC, LCS and CE groups were randomly selected and dissected. Then the midguts were removed and immediately put into Bouin's fixative solution for 24 h. Midguts were further processed according to the method of Li et al. [14]. Then, an inverted phase contrast microscope (Olympus, Japan) was used to examine and photograph for sections. The villus number and villus height were quantified within six different fields, randomly visually selected from five replicates in each group. Pictures of the sections were analyzed using software Digimizer. The survival rate (SR; %), feed conversion ratio (FCR) and specific growth rate (SGR; %/d) were calculated using the following formulas:

$$SR = N_t \times 100/N_0$$

$$FCR = F_0/WG$$

$$SGR = (\ln W_t - \ln W_0) \times 100/t$$

where, W_t and W_0 denote the final and initial weight of shrimp respectively; t was the duration of experiment days; N_t and N_0 denote the final and initial number of shrimp respectively. F_0 is the feed offered (dry weight) and WG is the weight gain (wet gain).

2.6. Immune genes expression

After the 42-day feeding trial, the hepatopancreas of 15 shrimp from each group were collected and immediately put into 2.0-mL sterile tubes containing RNA Keeper Tissue Stabilizer (Vazyme, China). Total RNA was extracted from the hepatopancreas using Trizol Reagent (Tiangen, China) according to the manufacture's protocol. The total RNA of each samples were examined by 1.5% agarose gel electrophoresis, Nanodrop 2000 (Thermo Scientific, USA) was used to quantitate the concentration of total RNA. Total RNA were reversely

Table 1
Sequences of the primers used in real-time qPCR.

Primer name	Sequence (5'-3')	Source or GenBank accession number
F-actin	GAGCAACACGGAGTTCGTTGT	Sun et al. [15]
R-actin	CATCACCAACTGGGACGACATGGA	
F-SOD	AGC CAA TGA CGT AAG CG	Sun et al. [15]
R-SOD	ACC ATC ACA AGA AAC CC	
F-LZM	TGT TCC GAT CTG ATG TCC	Sun et al. [15]
R-LZM	GCT GTT GTA AGC CAC CC	
F-proPO	TCC ATT CCG TCC GTC TG	Sun et al. [15]
R-proPO	GGC TTC GCT CTG GTT AGG	
Relish-F	GAGGTATGGTCAGGGTATGGTG	Ge et al. [16]
Relish-R	ATTCTTCTGCGTTTCAAGGTGT	
Toll-F1	TGGACTTCTGCTCGGACAAC	DQ923424
Toll-R1	GTACATGTCCTTGGTCGGCA	
Imd-F2	TCACATTGGCCCCGTTATCC	FJ592176
Imd-R2	ATCTCGCGACTGCACTTCAA	
TOR-F	TGCCAACGGGTGGTAGA	Duan et al. [17]

transcribed to cDNA by HiScript® II Q RT reagent Kit (Vazyme, China).

Real-time quantitative RT-qPCR was performed on qTOWER 2.2 (Analytikjena, Germany). The PCR amplification program, designed according to the specification of ChamQ™ SDCR® Color qPCR Master Mix (Vazyme, China) was operated at 95 °C for 30s, then 40 cycles of 95 °C for 10s, 60 °C for 30s, 95 °C for 15s, 60 °C for 60s, and 95 °C for 15s. The reaction was carried out with three replicates of each sample. The genes of superoxide dismutase (SOD), lysozyme (LZM), prophenoloxidase (proPO), Toll, Immune deficiency (Imd), Relish, TOR, 4E-BP, eIF4E1α and eIF4E2 were selected to response the effects of diets supplemented with different additives on growth and immunity of shrimp. The primers of Toll, immune deficiency (Imd), Relish, TOR, 4E-BP, eIF4E1α and eIF4E2 genes were listed in Table 1. The $2^{-\Delta\Delta Ct}$ comparative CT method was employed to quantitate expression levels for Toll, immune deficiency (Imd), Relish, TOR, 4E-BP, eIF4E1α and eIF4E2 genes relative to the β-actin gene.

2.7. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) in SPSS 21; mean differences among treatments were compared using Duncan's multiple-range test. The significance level was set at $p < 0.05$. Before statistical analyses, data were checked for normality of distribution and homogeneity of variance with the Kolmogorov-Smirnov test and Levene's test, respectively. If the data did not conform to normality of distribution and homogeneity of variance, the data were transformed. Data were expressed as means ± SEM.

3. Results

3.1. Growth performance of shrimp

The survival rates of shrimp ranged from 91.67% to 96.67%, and no significant difference was found among all treatments ($P > 0.05$). The final weight and specific growth rate of shrimp in DS, LC, LCS and CE groups were all significantly higher than those in the control ($P < 0.05$). The feed conversion ratio of the DS, LC, LCS and CE groups were all significantly lower than that of the control ($P < 0.05$; Table 2).

3.2. Intestinal histological analysis

The mid-intestine histological analysis results of shrimp in the control, FS, DS, LC, LCS and CE groups were exhibited in Table 3 and Fig. 1. As shown in Table 3, the villus height of mid-intestine in DS, LC, LCS and CE groups were significantly higher than that in the control ($P < 0.05$) and there was no significant difference among the DS, LC, LCS and CE groups ($P > 0.05$). Intestinal wall thickness of mid-intestine in the FS, DS, LC and LCS groups were all significantly higher than that in the control ($P < 0.05$), whereas that of FS, DS, LC and LCS groups were statistically same.

3.3. Challenge test

As shown in Fig. 2, the disease resistance of *L. vannamei* to *V. parahaemolyticus* challenge was changed after diets were supplemented with different additives. The cumulative mortality of shrimp in the DS, LC, LCS and CE groups was significantly lower than that in the control group ($P < 0.05$); however, no significant difference was observed between the FS group and the control ($P > 0.05$).

3.4. Expression levels of LZM, proPO and SOD genes

Effects of diet supplemented with different additives on LZM, proPO and SOD genes in shrimp were shown in Fig. 3. Relative expression levels of the LZM, proPO, SOD genes in the DS and LCS groups were significantly higher than those in the control ($P < 0.05$). Relative expression level of LZM gene in the FS, DS, LC, LCS and CE groups was all significantly higher than that in the control ($P < 0.05$). Compared to the FS, LC and CE groups, relative expression level of LZM gene in the DS and LCS groups increased significantly ($P < 0.05$), whereas it was statistically same between the DS and LCS groups ($P > 0.05$). Relative expression level of proPO gene in the DS, LC, LCS and CE groups was significantly higher than that in the control ($P < 0.05$). The relative expression levels of LZM, SOD and proPO genes in LCS group was the highest, while the relative expression levels of LZM and SOD genes were statistically same between DS and LCS groups ($P > 0.05$).

3.5. Expression levels of Toll, Imd and Relish genes

Compared with the control, the relative expression level of Toll gene increased significantly in the DS and LCS groups ($P < 0.05$), whereas that of the DS and LCS groups was statistically the same ($P > 0.05$). Relative expression level of Imd gene in the DS, LCS and CE groups were significantly higher than that in the control ($P < 0.05$). The relative expression level of Relish gene in the DS, LC and LCS groups was significantly higher than that in the control ($P < 0.05$). Relative expression levels of Toll, Imd and Relish genes were the highest in the DS, LCS and DS groups, respectively, while that of Toll, Imd and Relish genes were statistically same between the DS and LCS groups. However, the relative expression levels of Toll, Imd and Relish genes in the FS group were not significantly different from the control ($P > 0.05$) (Fig. 4).

3.6. Expression levels of mTOR signaling pathway genes

The relative expression levels of mTOR signaling pathway genes in the hepatopancreas of shrimp were exhibited in Fig. 5. Compared with the control, the relative expression level of the TOR gene was significantly increased in the FS, DS, LC, LCS and CE groups ($P < 0.05$). The relative expression level of TOR gene in LCS group was significantly higher than that in the FS, DS, LC and CE groups ($P < 0.05$). Relative expression level of the 4E-BP gene in the FS, DS, LC, LCS and CE groups was significantly higher than that in the control ($P < 0.05$); relative expression level of 4E-BP gene was the highest in LC group, while the relative expression level of 4E-BP gene in the DS, LC and LCS groups was statistically same ($P > 0.05$). The relative expression level of eIF4E1α gene was significantly increased in DS, LC, LCS and CE groups. The relative expression level of eIF4E1α gene was the highest in LCS group, while the relative expression level of eIF4E1α gene in the DS, LC, LCS and CE groups was statistically same ($P > 0.05$). The relative expression level of eIF4E2 gene was significantly increased in DS, LC, LCS and CE group ($P < 0.05$). The relative expression level of

Table 2
Growth performance of shrimp in different treatments.

Index	Treatment						ANOVA *P*
	Control	FS	DS	LC	LCS	CE	
SR%	95.00 ± 3.33^{a}	91.67 ± 3.73^{a}	93.33 ± 3.12^{a}	96.67 ± 2.04^{a}	96.67 ± 2.04^{a}	91.67 ± 2.64^{a}	0.676
Initial weight/g	3.73 ± 0.05^{a}	3.82 ± 0.03^{a}	3.84 ± 0.04^{a}	3.82 ± 0.02^{a}	3.75 ± 0.05^{a}	3.77 ± 0.05^{a}	0.651
Final weight/g	6.12 ± 0.27^{a}	6.62 ± 0.09^{a}	8.27 ± 0.14^{bc}	8.65 ± 0.06^{c}	8.31 ± 0.21^{bc}	7.92 ± 0.23^{b}	0.000
SGR%/d	1.23 ± 0.08^{a}	1.37 ± 0.05^{a}	1.92 ± 0.06^{b}	2.04 ± 0.02^{b}	1.99 ± 0.05^{b}	1.85 ± 0.08^{b}	0.000
FCR	1.74 ± 0.15^{a}	1.68 ± 0.07^{a}	1.21 ± 0.03^{bc}	1.05 ± 0.04^{c}	1.19 ± 0.05^{bc}	1.37 ± 0.06^{b}	0.000

Data with different letters at the same row meant significant difference with each other ($P < 0.05$). Data were expressed as mean ± S. E. M. (n = 5). SR, survival rate; FCR, feed conversion ratio; SGR, specific growth rate; Control, commercial feed; FS, commercial feed supplemented with fermentation supernatant; DS, commercial feed supplemented with spray-dried spores; LC, commercial feed supplemented with live *C. butyricum*; LCS, commercial feed supplemented with live cell and supernatant; CE, commercial feed supplemented with cell-free extract.

Table 3
Mid-intestine histological results in the control, FS, DS, LC, LCS and CE groups.

Treatment	Villus height/μm	Intestinal wall thickness/μm
Control	28.9 ± 0.59^{a}	23.2 ± 0.75^{a}
FS	27.8 ± 1.3^{a}	43.5 ± 2.15^{b}
DS	36.3 ± 0.69^{b}	47.1 ± 1.59^{b}
LC	36.7 ± 0.45^{b}	42.7 ± 2.11^{b}
LCS	35.8 ± 0.65^{b}	45.5 ± 1.29^{b}
CE	34.2 ± 1.03^{b}	19.3 ± 0.84^{a}

Data with different letters at the same row meant significant difference with each other($P < 0.05$). Data were expressed as mean ± S. E. M. Control, commercial feed; FS, commercial feed supplemented with fermentation supernatant; DS, commercial feed supplemented with spray-dried spores; LC, commercial feed supplemented with live *C. butyricum*; LCS, commercial feed supplemented with live cell and supernatant, CE, commercial feed supplemented with cell-free extract.

eIF4E2 gene was the highest in LC group, while the relative expression level of eIF4E2 gene in the DS, LC and LCS groups was statistically same ($P > 0.05$).

4. Discussion

Probiotics offer a promising alternative to chemicals and antibiotics in aquatic animals, aiding in the protection of cultured species [18]. A wide range of micro-organisms has been employed in aquaculture, in which Gram-positive and Gram-negative bacteria are applied effectively [8]. Other nonbacteria candidates such as bacteriophages, microalgae and yeasts have also been explored frequently as probiotics for use in aquaculture [8]. As a dietary probiotic, *C. butyricum* has been widely applied in livestock and poultry farming [19,20], however, only few results on specific aquatic animals, such as *Miichthys miiuy* and *L. vannamei*, etc., have been reported thus far [10]. However, there is also scarce information regarding this probiotic's efficacy as an FS, LC, DS, CE or LCS dietary supplement in *L. vannamei*. Most of probiotics have been found to be positive promoters of aquatic animal growth, feed utility, survival and health [8]. Song et al. [21] reported that the growth performance of *M. miiuy* was improved after diet supplemented with live *C. butyricum*. Research from Duan et al. [10] and Li et al. [11] revealed that the spores or live cells of *C. butyricum* could improve growth performance and resistance against ammonia stress and *V. parahaemolyticus*, respectively. In this study, diet supplemented with spores, live cells, mixture of live cells and supernatant and cell-free extract of *C. butyricum* CBG01 significantly improved the growth performance and disease resistance of shrimp. However, compared with the control, the fermentation supernatant exerted no obvious change in the growth performance and disease resistance of shrimp. These results were in agreement with Pan et al. [22], who discovered that fish fed live or dead *C. butyricum* demonstrated low mortality after infection with *V. anguillarum* or *Aeromonas hydrophila*; however, no effect was found in the fermentation supernatant group.

The intestine plays a key role in the growth and health of aquatic animals. The surface area of intestine was directly related to the digestion and absorption of food. Intestinal villus provided a vast absorptive surface area, and an increase in villus height could improve nutrient absorptive ability [23]. Duan et al. [10] reported that intestine epithelium height was increased after diet supplemented with *C. butyricum* in shrimp's feed. An increase in intestinal wall thickness contributed to movement, causing intestinal content to mix with various digestive enzymes and immune enzymes, which was also related to the digestion and absorption of food [24]. In this experiment, the villus height and intestinal wall thickness in the DS, LC and LCS groups and villus height in the CE group increased significantly ($P < 0.05$). The surface area of the shrimp's intestine increased significantly when supplemented with the spores, live cells, and mixture of live cells and supernatant and cell extract of *C. butyricum*. These findings suggest that an enhanced specific growth rate and reduction in feed conversion ratio for shrimp could be partially attributed to the positive changes in intestinal morphology.

As a signal transduction pathway, the mTOR signaling pathway, which plays a vital role in nutrition regulation and has complex impact on cell growth, widely exists in eukaryotes [25], food intake [26] and environmental stresses [27]. Target of rapamycin (TOR), as a highly conserved protein kinase, is well known to initiate translation and stimulate protein synthesis via ribosomal protein S6 kinase-polypeptide 1 (S6K1) and eukaryotic translation initiation factor 4E-binding proteins (4E-BPs). In mammalian cells, 4E-BP as a downstream effector of TOR participates in the regulation of mTORC1 (mTOR complex 1) and promotes protein synthesis [28]. mTORC1 was sensitive to rapamycin and integrates inputs from at least five major signals (i.e., growth factors, genotoxic stress, energy status, oxygen, and amino acids) to regulate many processes involved in the promotion of cell growth and proliferation [29]. The eIF4E family of proteins consists of three large subfamilies (i.e., eIF4E1, eIF4E2, and eIF4E3), and most eIF4E proteins possess at least some 5′-cap binding activity [30]. In the mTOR signaling pathway, the activity of the eIF4E protein is regulated by 4E-BP [29]. In addition, Piron et al. [31] reported that the role of eIF4E in resistance to two potyviruses, Potato virus γ (PVY) and Tobacco etch virus (TEV), was demonstrated by molecular cloning of the recessive resistance gene pot-1 in tomato; therefore, the eIF4E protein not only is essential for cell growth and is related to organism immunity [31]. The eIF4E protein not only is essential for the growth of cells but also relate to immunity of organism [31]. TOR pathway is a key regulator of the balance between protein synthesis and degradation in response to nutrition quality and quantity [32,33], and the protein synthesis is essential for cell growth, proliferation, apoptosis, and autophagy [34]. Moreover, immune protein synthesis and nutrient transport are also each related to mTOR [35]. In this study, higher genes expression of the mTOR signaling pathway was induced in the DS, LC, CE and LCS groups; however, no up-regulated gene expression of mTOR-related

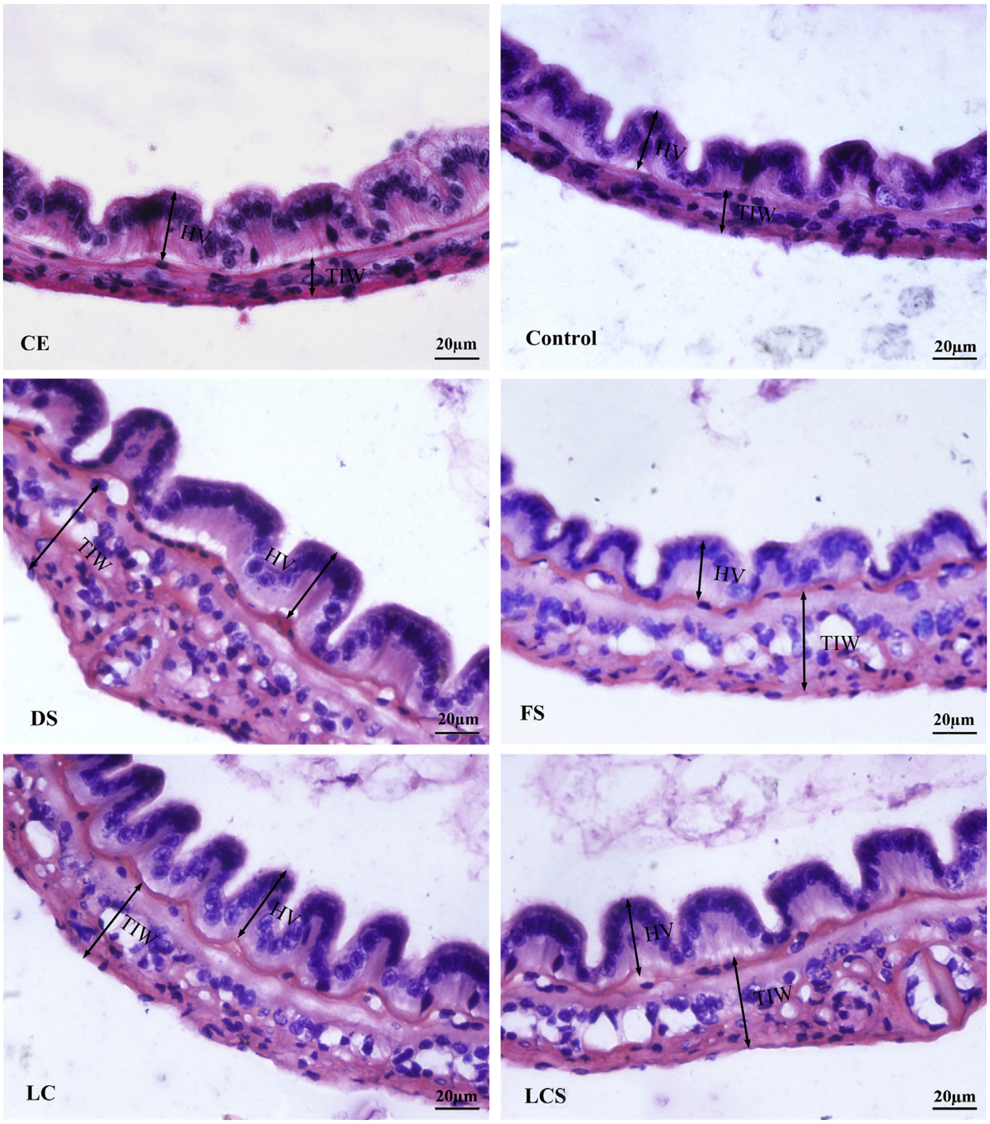

Fig. 1. Transversal section photomicrographs of shrimp's mid-intestine. HV, villus height; TIW, intestinal wall thickness.

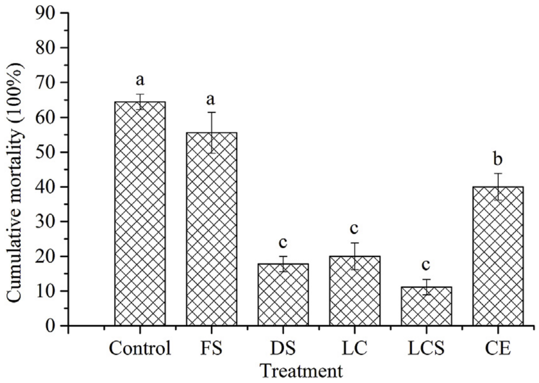

Fig. 2. Cumulative mortality of shrimp in different treatments. Data were expressed as mean ± S. E. M. Data with different letters at the column indicated significant difference with each other ($P < 0.05$).

genes was observed except for the 4E-BP gene in the FS group. In view of the growth performance of shrimp, the final weight and SGR of shrimp were promoted in the DS, LC, CE and LCS groups and FCR declined significantly. By contrast, no enhancement in growth performance was found in the FS group. These results imply that the mTOR signaling pathway is closely associated with shrimp growth.

L. vannamai, as an invertebrate, relies on the innate immunity to defend against microbial invaders by activating various immune genes [36], including cellular and humoral systems. The cellular and humoral systems are not independent but rather interactive and interdependent to synergistically protect the host and eliminate foreign particles and pathogens in shrimp [37]. As the first line of defense against pathogens, humoral responses are crucial aspects of the innate immune system [38]. The synthesis and release of several immune proteins, such as proPO, SOD and LZM, are partially important responses to invading pathogens [37]. The proPO-activating system serves an important role in converting zymogen proPOs to functionally active phenoloxidases, which subsequently catalyze the formation of quinone reactive intermediates for melanin synthesis at injury sites or around invading microorganisms [39,40]. The expression level of proPO gene is closely related to the activity of corresponding enzymes and associated with the cumulative mortality of shrimp when challenged with pathogens [41]. LZM and SOD are also the main components of the humoral system against pathogens. LZM can lyse the cell wall of both Gram-positive and Gram-negative bacteria and shows a strong inhibition against the shrimp pathogens *V. alginolyticus* and *V. parahemolyticus* [42]. The function and significance of LZM have been demonstrated in shrimp [42], such as *Marsupenaeus japonicus*, *P. monodon*,

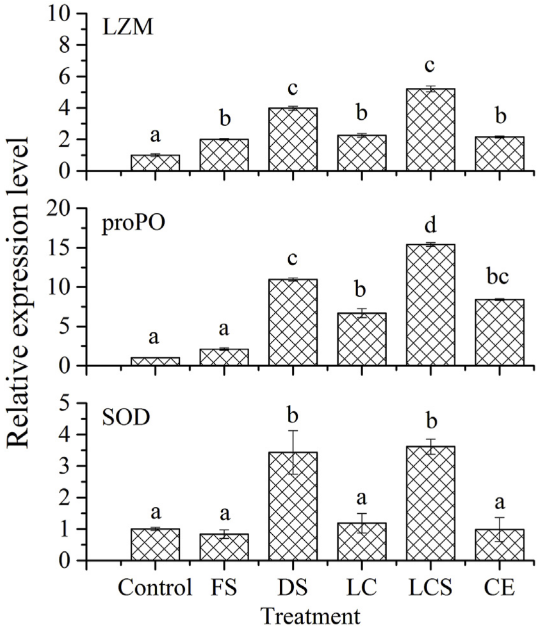

Fig. 3. Effects of diet supplemented with different additives on relative expression levels of LZM, proPO and SOD genes in the hepatopancreas of *L. vannamei*. β-Actin was the reference gene. Data were expressed as mean ± S. E. M. Data with different letters at the column indicated significant difference with each other ($P < 0.05$).

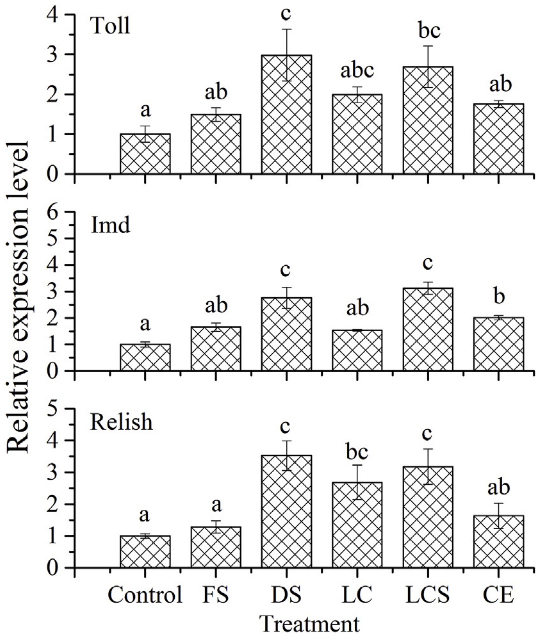

Fig. 4. Effects of diet supplemented with different additives on relative expression levels of Toll, Imd and Relish genes in the hepatopancreas of *L. vannamei*. β-Actin was the reference gene. Data were expressed as mean ± S. E. M. Data with different letters at the column indicated significant difference with each other ($P < 0.05$).

Fenneropenaeus merguiensis, *L. stylirostris*, *F. chinensis*, and *L. vannamei*. SOD can eliminate reactive oxygen species produced under the stress of temperature, hypoxia, pH, toxicant, and pathogens in the host to sustain cell homeostasis [43]. In this study, the relative expression levels of proPO, LZM and SOD were significantly promoted in the hepatopancreas of shrimp for the DS and LCS groups ($P < 0.05$). These findings suggest that the spores of *C. butyricum* and mixture of live cells and supernatant in the diet could serve as effective immuno-stimulating substances for producing proPO, LZM, and SOD to protect shrimp against pathogens.

Antimicrobial peptides (AMPs), which play an important role in killing or controlling the microorganisms and modulating other immune responses, are primary humoral immune effectors [44]. Studies have confirmed the significance of AMPs in the innate immune system of shrimp [42], such as *L. vannamai*, *F. chinensis*, *L. setiferus*, and *Penaeus monodon*. According to Hoffmann et al. [45], AMP genes are regulated by the Toll and Immune deficiency (Imd) pathways. The Toll pathway can be activated by Gram-positive bacteria and fungi, whereas the Imd pathway responds to Gram-negative bacteria infection [45]. When pathogenic microorganisms invade the host, receptors of Toll or Imd specifically recognize the relative molecules of pathogens and generate a signal that is transferred to activate the cell nuclear transcription factors (NF-κB) via a series of complex cascade reactions [45]. NF-κB transcription factors play important roles in mediating immune and inflammatory responses [46]; these transcription factors include Relish, Dorsal, and Dif, which are associated with the Toll and Imd pathways [47]. When a host is infected by Gram-negative bacteria, the Relish gene will be activated to induce expression of antibacterial peptides and other immuno-related genes, a polyphenol oxidase cascade reaction and the corresponding inflammatory cascade reaction [48]. Because the mechanisms of the Toll and Imd pathways are illuminated in *Drosophila* [49], the two pathways have been also confirmed in shrimp, such as *Exopalaemon carinicauda* [50], *F. chinensis* [50], *P. monodon* [51], and *L. vannamei* [52]. In this study, diets supplemented with the spores of *C. butyricum* and mixture of live cells and supernatant significantly improved the expression of Toll, Imd and Relish genes, whereas single live cells only significant enhanced the expression of Relish gene in shrimp.

According to the FAO/WHO, probiotics are defined as live microorganisms which, when administered in adequate amounts confer a health benefit on the host. However, some published data indicate that the use of non-viable microbial cells or cell components can also confer a benefit on the host (i.e., paraprobiotics, also known as ghost probiotics [53,54]). Unfortunately, the advantages of non-viable microbes over their viable counterparts represent a hotly debated topic in aquaculture. Dietary supplementation with heat-inactivated probiotics has stimulated the innate immune parameters in fish [55]. Diets with viable *Lactobacillus rhamnosus* induced a higher expression of the immune genes (i.e., TNF, TGF-β, IFN and Ig) than those with heat-killed probiotics in rainbow trout, *Oncorhynchus mykiss* [56]. Cellular components and viable cells of *Bacillus licheniformis* and *B. pumilus* increased the expression of LZM and respiratory burst of rohu [57]. Conversely, the addition of formalized, sonicated, heat-killed and cell-free supernatant of probiotics conferred less protection in rainbow trout against pathogens *Streptococcus iniae* and *Lactococcus garvieae* [58]. Zheng et al. [59] reported that live or dead *L. plantarum* and cell-free extract could improve the growth performance and immune responses of *L. vannamei*. So far, no comparison has been reported among *C. butyricum*'s efficacy as live cells, spores, cell-free extract, fermentation supernatant, and mixture of live cells and supernatant in *L. vannamei*, although live or dead *C. butyricum* had the same impact on the immune responses and disease resistance of *M. miiuy* [22]. Results from the present study showed that live cells, spores, cell-free extract, and mixture of live cells and supernatant of *C. butyricum* CBG01 significantly improved the growth performance and resistance to *V. parahaemolyticus* in shrimp. All immune-related and mTOR signaling-related genes expression levels were enhanced significantly in shrimp supplemented with the spores and mixture of live cells and supernatant of *C. butyricum*. The disease resistance and immune responses of shrimp supplemented with live cells and spores of *C. butyricum* were better than those receiving cell-free extract of the *C. butyricum* group. Interestingly, although diet supplemented with live *C. butyricum* could significantly improve the growth performance, disease resistance, mTOR signaling-related genes expression of shrimp, no up-regulated expression of proPO, LZM and

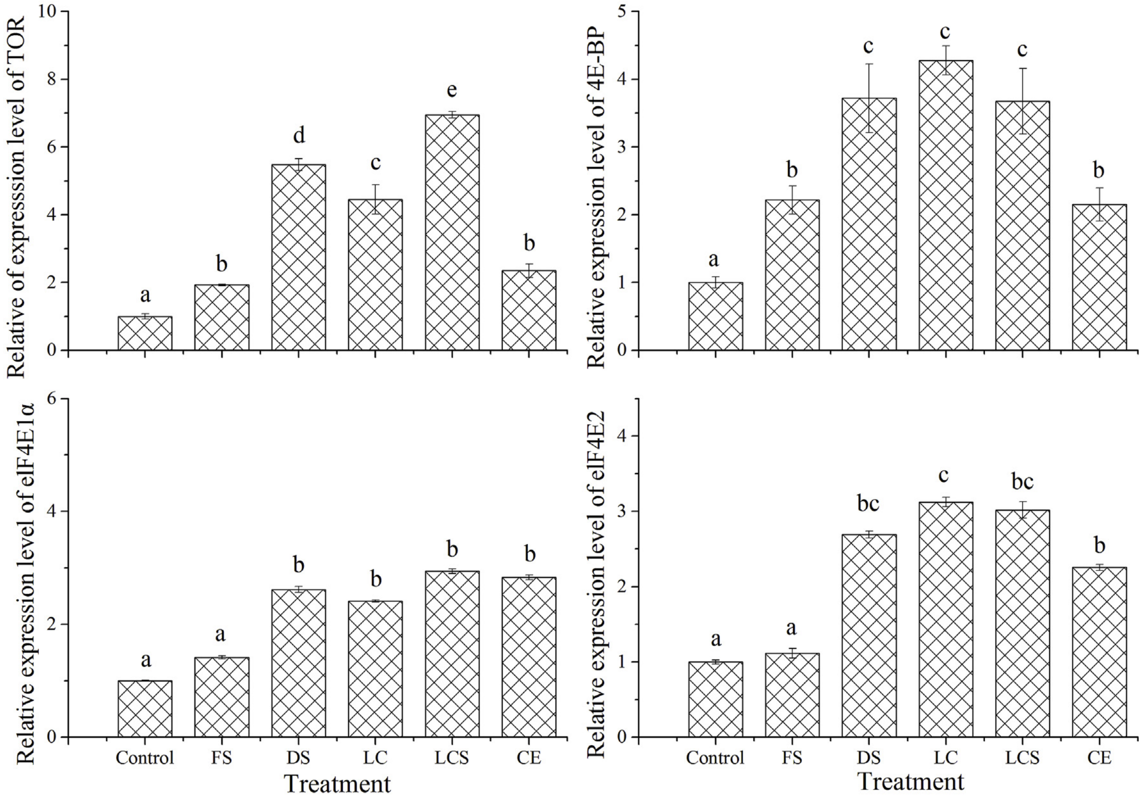

Fig. 5. Effects of diet supplemented with different additives on mTOR signaling-related gene expression levels in the hepatopancreas of *L. vannamei*. β-Actin was the reference gene. Data were expressed as mean ± S. E. M. Data with different letters at the column indicated significant difference with each other ($P < 0.05$).

SOD genes were observed; that is, i.e., live cells induced less immune response in shrimp. On the contrary, no significant difference was found in all parameters between the shrimp dietary supernatant and the control.

Despite extensive research, the mechanisms of probiotics in aquaculture are far from completely understood. Based on previous studies, the host's immune system could be influenced by different probiotic strains, thereby modulating the host's immune responses [53]. However, the action modes of various probiotics found to activate host immunity may be quite different among *Lactobacillus*, *Bifidobacterium*, *Bacillus*, and so on. Structural components of the cell play a major role in mediating immunomodulatory activity, particularly the cell envelope which includes cell wall constituents, S-layer proteins, capsule and pellicle, etc [60]. Some proteins associated with probiotic bacteria have been shown to elicit immune responses, such as, the heat-shock protein GroEL (Hsp60 class) of *L. johnsonii* La1 [61]. In addition, probiotics can interact with the host immunity via their genomic DNA (e.g., CpG in prokaryotic DNA) [62]. *C. butyricum* is a butyric-acid-producing and endospore-forming bacterium. The bacterial fermentation supernatant contains butyrate along with the residual medium and other kinds of extracellular products such as bacteriocin and lipoteichoic acid [63]. The metabolites produced by *C. butyricum* might play important roles in probiotic action in shrimp, although such metabolites exerted no significant positive effects separately. A mixture of live cells and supernatant of *C. butyricum* demonstrated its better probiotic abilities in growth performance, disease resistance and mTOR signaling-related genes expression of shrimp. Spores of *C. butyricum* also exhibited similar probiotic effects. Spores in this study were obtained via spray-drying technology after the spore forming rate was more than 95% in the liquid fermentation process, and 60% supernatant was removed by filtration before spray drying. Thus, some amount of metabolites of *C. butyricum* metabolites were also contained in the spray-dried spores. However, live cells unexpectedly induced less immune response in shrimp unexpectedly and only enhanced proPO, Imd and mTOR signaling-related genes expression in shrimp supplemented with cell-free extract. These results suggest that the integration of *C. butyricum* and their metabolites supplemented into feed could significantly improve growth performance, intestinal morphology, immunity capacity and resistance against *V. parahaemolyticus* in *L. vannamei*. Unfortunately, the achieved results of the present study are insufficient to unveil all the potential properties of the different probiotic components of *C. butyricum*. Therefore, further investigation into their mechanisms of action is required.

5. Conclusion

Diet supplemented with live cells, spray-dried spores, cell-free extract, and mixture of live cells and supernatant of *C. butyricum* CBG01 significantly improved the growth performance and disease resistance of *L. vannamei*., and had a positive effect on the intestinal morphological structure. The metabolites produced by *C. butyricum* might play important roles in probiotic action in shrimp, but demonstrated no significant positive effects on their own. Spray-dried spores and mixture of live cells and supernatant of *C. butyricum* exerted better probiotic benefits in modulating immune responses. Comparatively, live cells surprisingly induced less immune response in shrimp and only enhanced proPO, Imd, and mTOR signaling-related genes expression in the cell-free extract group. When growth performance, immune responses, disease resistance and shelf-life problems of probiotics were considered collectively, the spray-dried spores of *C. butyricum* CBG01 can be recommended for *L. vannamei* farming based on the results of this study. These results are essential to an understanding of the roles of *C. butyricum* in the health management of shrimp as well as in the effective and appropriate applications of probiotics in shrimp farming.

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