



# Effects of *Bacillus velezensis* Supplementation on the Growth Performance, Immune Responses, and Intestine Microbiota of *Litopenaeus vannamei*

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In the present study, *Bacillus velezensis* (BV007, CGMCC No. 20039) was isolated from the gut of *Litopenaeus vannamei*, and the effects of BV007 on the growth performance, immune responses, and intestine microbiota of the shrimp were investigated. A total of 1,200 healthy shrimp (3.0 ± 0.3 cm, 0.32 ± 0.8 g) were randomly divided into four groups, and fed diets supplemented with different levels of BV007 (C: 0; BV1: 1 × 10<sup>5</sup> CFU/g; BV2: 1 × 10<sup>7</sup> CFU/g; and BV3: 1 × 10<sup>9</sup> CFU/g) for 8 weeks. The results showed a significantly increased final body length (FBL), length gain rate (LGR), final body weight (FBW), weight gain rate (WGR), plumpness index (PI), and specific growth rate (SGR) in shrimp fed with BV007 for 42 days compared with shrimp fed with control diet. The activity of α-amylase in hepatopancreas was also significantly increased in the BV007-administered groups. After 42 days of growth trial, the challenge test with *Vibrio parahaemolyticus* was conducted for 2 weeks. The enhanced immune responses were exhibited by shrimp fed with BV007 after *V. parahaemolyticus* challenge, particularly in respiratory bursts and superoxide dismutase, catalase, and alkaline phosphatase activities. Moreover, the administration of BV007 could considerably increase the abundance of potential probiotics (*Bacillus*) and reduced the abundances of potential pathogenic bacteria (*Vibrio*) in shrimp intestines. In conclusion, the dietary supplementation with *B. velezensis* BV007 could promote the growth performance, enhance the immune responses, and modulate the intestine microbiota of shrimp, and 10<sup>7</sup> CFU/g feed was recommended to be used as a feed additive to enhance the growth and health status of shrimp.

**Keywords:** *Litopenaeus vannamei*, *Bacillus velezensis*, growth performance, innate immunity, intestinal health

## INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is one of the major cultivated aquaculture crustacean species worldwide. In recent years, shrimp aquaculture has developed rapidly and intensively, but the low growth rates and disease have occurred more frequently, causing considerable economic losses (Neiland et al., 2001). The use of hormones and antibiotics as a traditional way to prevent and treat infectious diseases has led to the evolution of pathogenic bacteria and environmental pollution. Great effort has been invested to develop the potential alternatives to antibiotics to avoid the creation of bacterial resistance and high antibiotic residues. Currently, probiotics, prebiotics, and medicinal herbs have shown to be a vital alternative additive for the improvement of host health (Raman et al., 2019; Dawood et al., 2020; Zhang et al., 2021).

Probiotics are valuable in animal production due to their improvement of feed value, enzymatic contribution to digestion, and activation of the immune responses (Gao et al., 2018; Wang Y. et al., 2019; Qin et al., 2020). For example, oral administration of *Bacillus* PC465 enhanced the growth performance and survival rate of *L. vannamei* (Chai et al., 2016). Dietary supplementation with *B. licheniformis* at  $10^5$  CFU/ml for 8 weeks enhanced abalones growth and enhanced disease resistance to *Vibrio parahaemolyticus* (Gao et al., 2018). Moreover, the activities of glutamic-pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) were significantly lower in shrimp fed with *Enterococcus faecalis* supplemented groups as compared with control shrimp (Wang Y. et al., 2019). In addition, dietary *Bacillus amyloliquefaciens* A23 at  $10^8$  CFU/g significantly enhanced the intestinal microbial diversity of *Procambarus clarkii* (Xu et al., 2021). Besides the promotion of *Lac. pentosus*, BD6 feeding can increase the relative abundance of beneficial bacteria and reduce the abundance of harmful pathogenic bacteria in the gut flora of shrimp, thus regulating the host immune system (Lin et al., 2017).

Although many studies suggest that *B. subtilis* possesses a number of beneficial traits when used as a supplement in the shrimp diets (Liu et al., 2010; Fu et al., 2011; Zokaeifar et al., 2012, 2014; Interaminense et al., 2018, 2019), few studies have investigated the appropriate dose of *B. velezensis* in *L. vannamei* farming. Therefore, the current study aimed to find the optimum supplementation level of *B. velezensis* and to investigate the potential effect on the growth performance, feed utilization, immune responses, and gut microbiota of *L. vannamei*.

## MATERIALS AND METHODS

### Bacterial Strain and Diet Preparation

*Bacillus velezensis* (named as BV007) was previously isolated from healthy *L. vannamei* intestines. The intestines were immersed in sterile phosphate buffered saline (PBS) (0.05 M, pH 7.4), shook, and eluted at 30°C for 30 min. Spread the eluate on LB nutrient agar (Beijing Land Bridge Tech. Co. Ltd.) plates with gradient dilution and incubated at 30°C for 24 h. After picking a single colony and streaking for purification, the strain 007 with a wide range of enzyme-producing abilities was obtained.

The genetic characterization of *B. velezensis* was accomplished by using 16S rDNA sequence analysis, and the strain was deposited by the China General Microbiological Culture Center (CGMCC no. 20039).

In the diet preparation, the bacterial strains were inoculated in nutrient broth, collected in sterilized water, and adjusted to  $10^9$  CFU/ml before feed production. The ingredients of the basal diet were measured to contain 41.35% crude protein and 7.67% crude lipid with fish meal, corn gluten meal, and soybean meal as the fundamental protein source whereas the main lipid sources were soybean oil and soy lecithin oil. Three experimental diets were supplemented with different levels of BV007:  $10^5$  CFU/g (BV1),  $10^7$  CFU/g (BV2), and  $10^9$  CFU/g (BV3). The group without BV007 was served as a control (C). The bacterial amounts of experimental diets were also confirmed by the plate count method, and the colonies that emerged on the plates were counted. The four formulated experimental diets were extruded through a 1.2-mm die. The resulting pellets were dried at 25°C with the aid of an air conditioner. After drying, all diets were stored at 4°C until usage.

### Experimental Design and Daily Management

Healthy *L. vannamei* (body length:  $3.0 \pm 0.3$  cm, body weight:  $0.32 \pm 0.8$  g) were purchased from a local farm (Yantai, Shandong Province, China) and acclimated at 25–27°C and 30‰ of salinity for 2 weeks before processing. The shrimp were randomly dispersed into 12 fiberglass tanks (60 L), each containing 100 shrimp. The shrimp were fed four times daily (06:00, 12:00, 17:00, and 22:00) at 6% of body weight. The cumulative mortality of the shrimp was recorded daily. After 42 days of feeding, the total number of shrimp and the weight in each tank was quantified to calculate the survival rate (SR, in percentage), final body weight (FBW), final body length (FBL), plumpness index (PI), specific growth rate (SGR, %/d) according to the methods described previously (Velmurugan et al., 2015; Amoah et al., 2019b).

### Sample Collection

Hemolymph samples (eight replicates were tested in each treatment, and three shrimps were tested in each replicate [ $n = 24$ ]) were individually withdrawn from the pericardial cavity of the shrimp using a 1-ml sterile syringe containing an anticoagulant (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA at pH 7.55 with an osmolality adjusted to 780 mOsm/kg with glucose). The volume ratio of anticoagulant to hemolymph was 1:1. The harvested hemocytes were centrifuged at 4°C, 4,000 rpm/min for 10 min, collected, and adjusted to  $10^6$  cells/ml with PBS (0.05 M, pH 7.4) for the phagocytic activity and respiratory burst assays. In addition, the hepatopancreas was collected for the assay of digestive, antioxidant, and immune-related enzyme activities, eight replicates were tested in each treatment, and three shrimps were tested in each replicate ( $n = 24$ ). The middle intestines and hepatopancreas of each group were collected sterile into 4% paraformaldehyde and stored at 4°C for the analysis of microorganisms (three parallel assays).

## Phagocytosis Assay

The phagocytosis assay was adapted from the method of Delaporte (Delaporte et al., 2003) with minor modifications. Briefly, the hemolymph was mixed with 2.3% yellow-green FluoSpheres (diameter 2.0  $\mu\text{m}$ , Polyscience, Eppelheim, Germany), and incubated in the dark at 18  $^{\circ}\text{C}$  for 1 h with rotation. After incubation, the hemocytes were analyzed by flow cytometry (BD Accuri C6 Plus, BD Biosciences, America) using the FL-1 tunnel to detect hemocytes containing fluorescent beads. The phagocytosis rates were expressed as the percentage of hemocytes that engulfed three or more beads.

## Respiratory Burst Activity

Respiratory burst activity was analyzed according to the protocols described by Kalgraff et al., 2011. Briefly, hemocytes ( $1 \times 10^6$  cells/ml) were challenged by phorbol 12-myristate 13-acetate (PMA; final concentration of 0.1  $\mu\text{g/ml}$ ) for 10 min. Subsequently, dihydrorhodamine 123 (DHR 123) was added to a final concentration of 2  $\mu\text{g/ml}$  and then incubated for 30 min prior to flow cytometry analysis. The flow cytometry analyses were done on a BD FACSCalibur flow cytometer equipped with a 15 mW 488 nm argon ion laser. Further data analyses were done using FCS Express 3 software (De Novo Software, CA, USA).

## Assay of Digestive and Immune-Related Enzymes

The digestive enzymes and immune enzymes were measured as described by Cao et al. (2012). Briefly, the digestive enzymes included  $\alpha$ -amylase (AMS), trypsin (TRP), lipase (LPS), glutamic-pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and the immune enzymes included catalase (CAT), superoxide dismutase (SOD), acid phosphatase (ACP), and alkaline phosphatase (AKP). The enzymatic activities were quantified with corresponding commercial detection kits (C016-1-1, A080-2-2, A054-1-1, C009-2-1, C010-2-1, A007-1-1, A001-3-2, A001-3-1, and A060-2-1, Nanjing Jiancheng Bioengineering Institute, P.R. China) according to the instructions of the manufacturer.

## Real-Time PCR Analysis

Total RNA was isolated from hepatopancreas of different treatments using RNAiso (TaKaRa, Japan) reagent. cDNA synthesis was performed according to Promega M-MLV RT Usage information. The temporal expression profiles were carried out with an Applied Biosystem 7500 fast real-time PCR system (Applied Biosystems, MA, USA) using SYBR Green I (Biotek, VT, USA). The PCR amplification program was 95 $^{\circ}\text{C}$  for 10 min, then 40 cycles of 95 $^{\circ}\text{C}$  for 15 s, 60 $^{\circ}\text{C}$  for 1 min, followed by 95 $^{\circ}\text{C}$  for 15 s, 60 $^{\circ}\text{C}$  for 1 min, and 95 $^{\circ}\text{C}$  for 15 s. At the end of each PCR, a dissociation curve analysis of amplification products was performed to confirm the purity of the PCR product. The  $2^{-\Delta\Delta\text{CT}}$  method was used to analyze the expression levels (Livak and Schmittgen, 2001). All data were given in terms of relative mRNA expressed as mean  $\pm$  SD ( $N = 6$ ). The primers for qRT-PCR of genes for antioxidant-related genes (*cat* and *sod*), antibacterial peptides genes (*crustin* and *penaeidin 3a*), pattern

recognition receptors (*lgbp* and *lec*), along with the housekeeping gene  $\beta$ -*actin*, are shown in **Table 1**.

## Histopathological Examination of the Intestine and Hepatopancreas

Three similar-sized shrimp from each tank were sampled and dissected to obtain the midgut and the hepatopancreas (5 mm<sup>3</sup>). The fixed intestinal and hepatopancreas samples were dehydrated in a graded series of ethyl alcohol and embedded in paraffin. Then, the paraffin blocks were sliced transversely into 5- $\mu\text{m}$  thick sections and stained with H&E. The villus length of intestinal slice and the cell type of hepatopancreas slice were measured by a light microscope with a computerized image system (Olympus, DP73, Tokyo, Japan).

## Intestinal Microbial Composition

The intestinal community of the micro-organisms was performed according to Suo (Suo et al., 2017) with slight changes. The total DNA of microbes in the intestine was extracted directly with the E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Inc., GA, USA) (Xin et al., 2015). The amplification and sequencing of the hypervariable region (V4 + V5) of the bacterial 16S DNA gene was performed for the sequencing analysis and species identification. High-throughput sequencing was performed on the IonS5TMXL platform (Novogene, China). The sequencing reads were assigned to each sample according to the individual unique barcode. The sequences were analyzed with the QIIME software package (Quantitative Insights Into Microbial Ecology) and the UPARSE pipeline (Caporaso et al., 2011). The reads were first filtered and clustered into operational taxonomic units (OTUs) at an identity threshold of 97%. The alpha and beta diversity analyses were then performed as described by Amoah et al. (2019b).

## Vibrio Parahaemolyticus Challenge

*Vibrio parahaemolyticus* was used for the challenge experiment (Amoah et al., 2019b). At the end of the culture experiment, 30 shrimps from each tank were injected with *V. parahaemolyticus* (final concentration  $2 \times 10^7$  CFU). In each tank, 20 shrimps were used for cumulative mortality assay, which was recorded daily for 14 days. The other 10 shrimps were cultured for immunoassays and 1 week after bacterial challenge, both the hemocytes and hepatopancreas were sampled and analyzed as described above. In addition, each treatment group was continuously fed with the experimental diets. The cumulative mortality rate was calculated following the formula of Liu et al. (2017).

## Statistical Analysis

The experimental data are presented as the mean  $\pm$  SE. The data were statistically analyzed for significance using a one-way ANOVA, and then the differences among the means at  $P < 0.05$  were tested with Duncan's multiple range test using SPSS 23.0 (SPSS Inc., 2005, IL, USA).

**TABLE 1** | Primers used to amplify immune-related genes in the present study.

| Primer           | Sequence information     | Accession number |
|------------------|--------------------------|------------------|
| cat F            | CATCCAGGATCGAGCAATCAA    | AY518322         |
| cat R            | TGAAGCCTGGCTCATCTTATC    |                  |
| sod F            | TGCCACCTCTCAAGTATGATTTC  | AB108065         |
| sod R            | TCAACCAACTTCTTCGTAGCG    |                  |
| crustin F        | GAGGGTCAAGCCTACTGCTG     | AY488497         |
| crustin R        | ACTTATCGAGGCCAGCACAC     |                  |
| pen 3 $\alpha$ F | CTCGTGGTCTGCCTGGTCTTCTTG | Y14926           |
| pen 3 $\alpha$ R | CAGGGCAACCGTTGTATGGA     |                  |
| lgbp F           | TGGACGCTTATGTCACCTAC     | AY249858         |
| lgbp R           | CTTCTACTTCATCTGTTGCT     |                  |
| lec F            | GATCGAGGACTGCGAAACCT     | BF024206         |
| lec R            | CCCCAGAAAGGTACACCCTG     |                  |
| $\beta$ -actin F | GAGCAACACGGAGTTCGTTGT    | AF300705         |
| $\beta$ -actin R | CATCACCAACTGGGACGACATGGA |                  |

pen 3 $\alpha$ , penaeidin 3 $\alpha$ ; lgbp, Lipopolysaccharide and  $\beta$ -1, 3-glucan binding protein; lec, lectin.

**TABLE 2** | Effects of different supplementation levels of *B. velezensis* BV007 on the growth performance and survival of *L. vannamei*.

| Items     | Treatments                        |                                  |                                   |                                  |
|-----------|-----------------------------------|----------------------------------|-----------------------------------|----------------------------------|
|           | C                                 | BV 1                             | BV 2                              | BV 3                             |
| FBL (cm)  | 9.25 $\pm$ 0.36 <sup>b</sup>      | 9.95 $\pm$ 0.41 <sup>a</sup>     | 9.9 $\pm$ 0.45 <sup>a</sup>       | 9.87 $\pm$ 0.40 <sup>a</sup>     |
| LGR (%)   | 167.96 $\pm$ 15.15 <sup>b</sup>   | 181.63 $\pm$ 10.42 <sup>a</sup>  | 183.63 $\pm$ 18.25 <sup>a</sup>   | 181.64 $\pm$ 9.92 <sup>a</sup>   |
| FBW (g)   | 4.78 $\pm$ 1.22 <sup>b</sup>      | 5.59 $\pm$ 0.40 <sup>a</sup>     | 5.58 $\pm$ 0.37 <sup>a</sup>      | 5.68 $\pm$ 0.29 <sup>a</sup>     |
| WGR (%)   | 1481.41 $\pm$ 112.47 <sup>b</sup> | 1781.66 $\pm$ 85.52 <sup>a</sup> | 1849.82 $\pm$ 122.80 <sup>a</sup> | 1837.64 $\pm$ 83.66 <sup>a</sup> |
| SGR (%)   | 16.60 $\pm$ 0.30 <sup>b</sup>     | 17.21 $\pm$ 0.26 <sup>a</sup>    | 17.37 $\pm$ 0.23 <sup>a</sup>     | 17.35 $\pm$ 0.23 <sup>a</sup>    |
| PI (g/cm) | 0.52 $\pm$ 0.03 <sup>b</sup>      | 0.57 $\pm$ 0.02 <sup>a</sup>     | 0.57 $\pm$ 0.03 <sup>a</sup>      | 0.58 $\pm$ 0.02 <sup>a</sup>     |
| SR (%)    | 84.71 $\pm$ 10.24                 | 86.64 $\pm$ 4.07                 | 80.41 $\pm$ 1.76                  | 78.53 $\pm$ 2.27                 |

IBL, initial body length; FBL, final body length; LGR, length gain rate; IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; PI, plumpness index; SR, survival rate; and CMR, cumulative mortality rate after infection. The values (mean of 10 replicates  $\pm$  SE) with different letters (a, b, c) represent statistically significant differences based on the LSD method ( $P < 0.05$ , one-way ANOVA).

## RESULT

### Growth Performance and Survival

The growth performance and survival of *L. vannamei* fed experimental diets are shown in **Table 2**. The results showed that FBL, FBW, PI, and SGR were significantly improved by the different levels of probiotics supplementation, whereas no significant differences were observed in SR among the control and probiotic diets.

### Phagocytic and Respiratory Burst Activity

After 42 days of feeding, there was a significant increase in the phagocytic activity of shrimp fed diets supplemented with *B. velezensis* as compared to the control group (**Figure 1A**). However, no significant increase in the rates of phagocytosis was found in either the probiotics-supplemented or non-supplemented shrimps after the *V. parahaemolyticus* challenge (**Figure 1B**). Compared with the control group, significantly reduced reactive oxygen species (ROS) production was observed in the BV3 group, but not in the BV1 or BV2 groups (**Figure 1C**).

In contrast, the production of ROS by hemocytes decreased statistically in the BV1, BV2, and BV3 groups when shrimp were challenged with *V. parahaemolyticus* ( $P < 0.01$ ; **Figure 1D**).

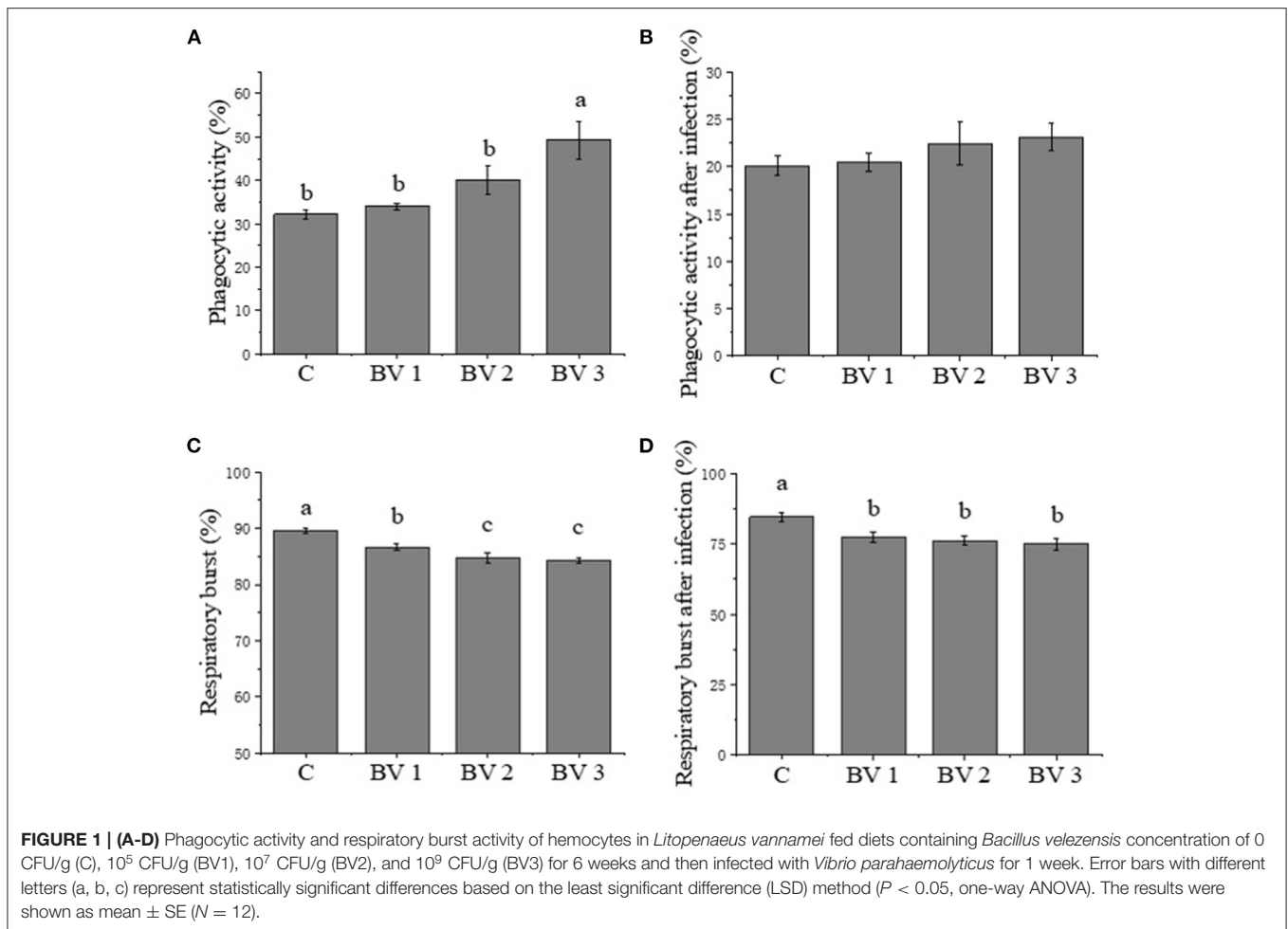
### Digestive Enzyme Activities

As shown in **Table 3**, the specific activity of the  $\alpha$ -amylase enzyme was enhanced in *B. velezensis*-supplemented groups. As compared with the control group, the AMS activity was enhanced significantly in shrimp fed BV1, BV2, and BV3 diets with the highest activity observed in the BV2 group ( $P < 0.01$ ). However, no significant differences were observed in TRP or LPS activities among any of the groups. In addition, the GOT and GPT activities were significantly reduced in the BV3 and BV1 groups as compared with the control group, respectively ( $P < 0.05$ ).

### Immune-Related Enzyme Activities

The activities of ACP showed no differences among the tested groups after being challenged with *V. parahaemolyticus* (**Table 4**). Similarly, no significant difference in AKP activity





**TABLE 3 |** Digestive enzyme activities in the hepatopancreas of *L. vannamei* fed diets containing 0 CFU/g (C), 10<sup>5</sup> CFU/g (BV1), 10<sup>7</sup> CFU/g (BV2), 10<sup>9</sup> CFU/g (BV3) *B. velezensis* for 6 weeks.

| Items          | Treatments                      |                                  |                                 |                                 |
|----------------|---------------------------------|----------------------------------|---------------------------------|---------------------------------|
|                | C                               | BV 1                             | BV 2                            | BV 3                            |
| LPS (U/gprot)  | 57.02 $\pm$ 4.33                | 57.28 $\pm$ 7.00                 | 57.28 $\pm$ 7.00                | 54.16 $\pm$ 5.20                |
| TRP (U/gprot)  | 117.57 $\pm$ 16.39 <sup>b</sup> | 122.62 $\pm$ 10.35 <sup>ab</sup> | 157.98 $\pm$ 12.32 <sup>a</sup> | 153.88 $\pm$ 13.88 <sup>a</sup> |
| AMS (U/gprot)  | 5.21 $\pm$ 0.15 <sup>b</sup>    | 5.62 $\pm$ 0.09 <sup>ab</sup>    | 5.94 $\pm$ 0.12 <sup>a</sup>    | 5.23 $\pm$ 0.17 <sup>b</sup>    |
| GPT (U/mgprot) | 97.61 $\pm$ 11.49 <sup>a</sup>  | 53.52 $\pm$ 6.07 <sup>b</sup>    | 66.93 $\pm$ 9.80 <sup>b</sup>   | 76.91 $\pm$ 12.88 <sup>ab</sup> |
| GOT (U/mgprot) | 38.86 $\pm$ 2.37 <sup>a</sup>   | 34.42 $\pm$ 3.83 <sup>ab</sup>   | 29.81 $\pm$ 4.51 <sup>ab</sup>  | 26.94 $\pm$ 1.76 <sup>b</sup>   |

The values (mean of 8 replicates  $\pm$  SD) with different letters (a, b, c) represent statistically significant differences based on LSD method ( $P < 0.05$ , one-way ANOVA). LPS, lipase; TRP, trypsin; AMS,  $\alpha$ -amylase; GPT, glutamic-pyruvic transaminase; GOT, glutamic oxaloacetic transaminase.

was detected among the BV1, BV2, and BV3 groups. However, the AKP activity was enhanced significantly after *V. parahaemolyticus* challenge among the tested groups ( $P < 0.01$ ; **Table 4**). Both the CAT and SOD activities were enhanced in *B. velezensis*-supplemented groups and in the groups challenged with pathogenic bacteria (**Table 4**). The highest SOD and CAT activities were observed in the BV2 ( $P < 0.01$ ; **Table 4**) and BV3 ( $P < 0.01$ ; **Table 4**) groups.

## Expression of Immune-Related Genes

As revealed in **Figure 2**, the feeding dosage of *B. velezensis* had a significant effect on the expression levels of immune-related genes. The expression levels of *cat* were significantly induced in BVs groups ( $P < 0.05$ , **Figure 2A**). After *V. parahaemolyticus* challenge, the *cat* transcripts in BV1 group were significantly higher than other groups ( $P < 0.05$ , **Figure 2A**). As concerned to *sod* expression, the higher expression levels were observed in BV2 and BV3 groups compared with C and BV1 groups, while

**TABLE 4** | Immune-related enzyme activities in the hepatopancreas of *L. vannamei* fed diets containing 0 CFU/g (C), 10<sup>5</sup> CFU/g (BV1), 10<sup>7</sup> CFU/g (BV2), or 10<sup>9</sup> CFU/g (BV3) *B. velezensis* for 6 weeks and then, infected with 10<sup>7</sup> CFU/ml *V. parahaemolyticus* for one week (use words with a superscript comma).

| Items                       | Treatments               |                          |                           |                           |
|-----------------------------|--------------------------|--------------------------|---------------------------|---------------------------|
|                             | C                        | BV 1                     | BV 2                      | BV 3                      |
| SOD (U/mgprot)              | 23.25±1.20 <sup>b</sup>  | 25.48±0.92 <sup>b</sup>  | 29.09±0.79 <sup>a</sup>   | 29.50±0.83 <sup>a</sup>   |
| SOD <sup>r</sup> (U/mgprot) | 17.99±1.56 <sup>b</sup>  | 21.05±0.85 <sup>ab</sup> | 23.57±1.57 <sup>a</sup>   | 21.64±0.77 <sup>a</sup>   |
| CAT (U/mgprot)              | 29.14±2.37 <sup>b</sup>  | 44.26±9.96 <sup>ab</sup> | 71.69±5.30 <sup>a</sup>   | 58.39±3.37 <sup>a</sup>   |
| CAT <sup>r</sup> (U/mgprot) | 23.62±2.43 <sup>b</sup>  | 49.70±6.64 <sup>a</sup>  | 59.82±5.75 <sup>a</sup>   | 51.05±8.50 <sup>a</sup>   |
| AKP (U/gprot)               | 22.77±3.83 <sup>b</sup>  | 28.55±5.19 <sup>ab</sup> | 36.39±3.76 <sup>a</sup>   | 29.30±4.32 <sup>ab</sup>  |
| AKP <sup>r</sup> (U/gprot)  | 65.31±2.38 <sup>b</sup>  | 88.13±7.26 <sup>ab</sup> | 93.37±7.59 <sup>a</sup>   | 86.79±7.47 <sup>ab</sup>  |
| ACP (U/gprot)               | 168.51±12.48             | 183.43±22.56             | 200.06±23.97              | 152.77±15.12              |
| ACP <sup>r</sup> (U/gprot)  | 115.39±3.83 <sup>a</sup> | 123.18±8.77 <sup>a</sup> | 138.98±10.48 <sup>a</sup> | 101.72±12.43 <sup>b</sup> |

The values (mean of 8 replicates ± SD) with different letters (a, b, c) represent statistically significant differences based on the LSD method ( $P < 0.05$ , one-way ANOVA). SOD, superoxide dismutase; CAT, catalase; AKP, alkaline phosphatase; ACP, acid phosphatase.

the levels were significantly upregulated in BV1 and BV2 groups after the bacterial challenge ( $P < 0.05$ , **Figure 2B**). No significant changes in the expression levels of antibacterial peptides (*crustin* and *penaeidin 3α*) were observed among C, BV1, BV2, and BV3 groups. After the bacterial challenge, the expression levels of *crustin* and *penaeidin 3α* in BV2 were significantly higher than the other groups ( $P < 0.05$ , Figures 2C,D). In addition, the expression levels of *Igbp* and *lec* were highly enhanced regardless of whether the shrimp were bacterial challenged, especially in BV1 and BV2 groups ( $P < 0.05$ , **Figures 2E,F**).

## Intestinal and Hepatopancreas Morphology

The intestinal and hepatopancreas morphometric parameters were presented in **Figure 3** and **Table 5**. The liver tubule of hepatopancreas in the BV addition groups are arranged relatively tightly and significantly generally star-shaped or polygonal ( $P < 0.05$ ), while the lumen of the control group is mostly circular. Besides, the control group distributed many differentiated E cells (embryonic cells), while the BV addition group distributed more B cells (secreting cells), F cells (fibrocyte), and R cells (absorbing cells). The shrimps from all dietary treatments had an intact epithelial barrier with extensive mucosal folds and abundant Microvilli. The mucosal structure of the control group was slightly loose, the length of intestinal villi was significantly shorter ( $P < 0.05$ ), and the number of mucosal epithelial cells was less. The intestinal tissue structures of the BV addition groups were relatively complete, and the length of the intestinal villi was increased and closely connected with the intestinal wall.

## Microbiota

### Richness and Diversity of Intestinal Microflora

The alpha diversity was calculated using the Shannon and Simpson indices and the Chao1, ACE, and PD whole tree estimators (**Table 6**). The nonparametric species-richness estimator Chao1 showed a minimum OTUs count of 287 in the BV1 group and a maximum count of 730 in the BV2 group ( $P < 0.05$ ). The highest level of supplemented probiotics (10<sup>9</sup> CFU/g) also had a higher OTU count than the control group ( $P < 0.05$ ). The number of observed species, Shannon

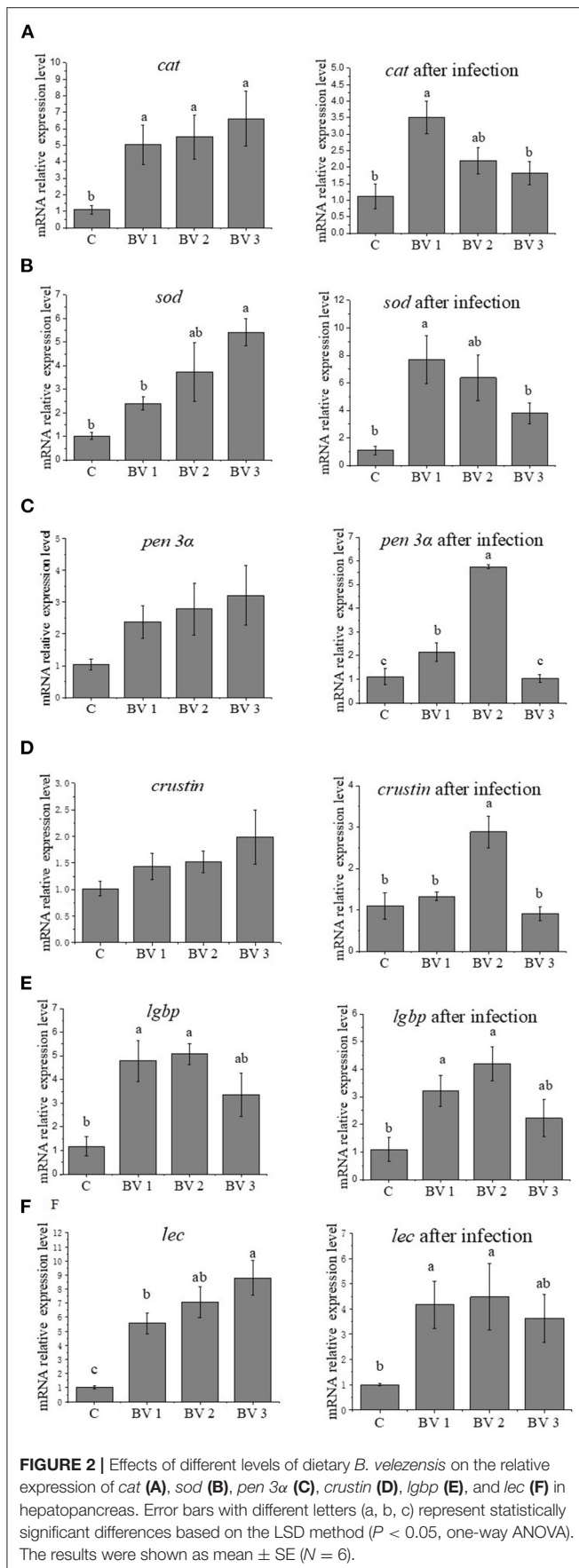
index, and ACE showed similar trends with BV3 exhibiting significantly higher values than the other groups. Phylogenetic diversity was measured using the PD whole tree estimator, which ranged from 26.50 to 52.64. All these indices suggested that the BV2 and BV3 groups had a higher microbial diversity in their intestines, whether measured *via* richness or evenness. A Venn diagram was constructed to identify the OTUs held in common or unique to shrimp under different diets. In this regard, 961 OTUs were shared among all the shrimp gut samples (**Figure 4**). In contrast, 43, 49, 197, and 245 OTUs were unique to the C, BV1, BV2, and BV3 diets, respectively. This observation suggests that the exposure of shrimp to different graded probiotics led to the selection of unique microbial populations.

### Bacterial Composition of Intestinal Microflora

The bacterial composition at the genus level in the intestine of *L. vannamei* is represented in **Figure 5**. The most abundant genera in the shrimp intestine were *Candidatus Bacilloplasma*, *Vibrio*, *Pseudoalteromonas*, *Bacillus*, and *Tenacibaculum*. After 42 days of feeding, the relative abundance of the predominant genera differed between the control and the groups fed with the BV2 and BV3 diets. In particular, the proportions of *Vibrio* and *Pseudoalteromonas* in the BV2 group (11.7 and 5.7%) were decreased compared with the C group (30.2 and 10.9%), BV1 group (15.2 and 10.7%), and BV3 group (17.9 and 8.8%). In addition, the proportion of *Bacillus* in the BV2 group (7.7%) was significantly higher than that in the C group (0.2%,  $P < 0.05$ ).

### Challenge Test

After the 2-weeks challenge with *V. parahaemolyticus*, the cumulative mortality rates of *L. vannamei* were shown in **Figure 6**. It was observed that the cumulative mortality was significantly lower in the treated groups than in the untreated ones, that is, 97.2, 61.1, 30.6, and 51.4% for shrimps fed with the C, BV1, BV2, and BV3, respectively.

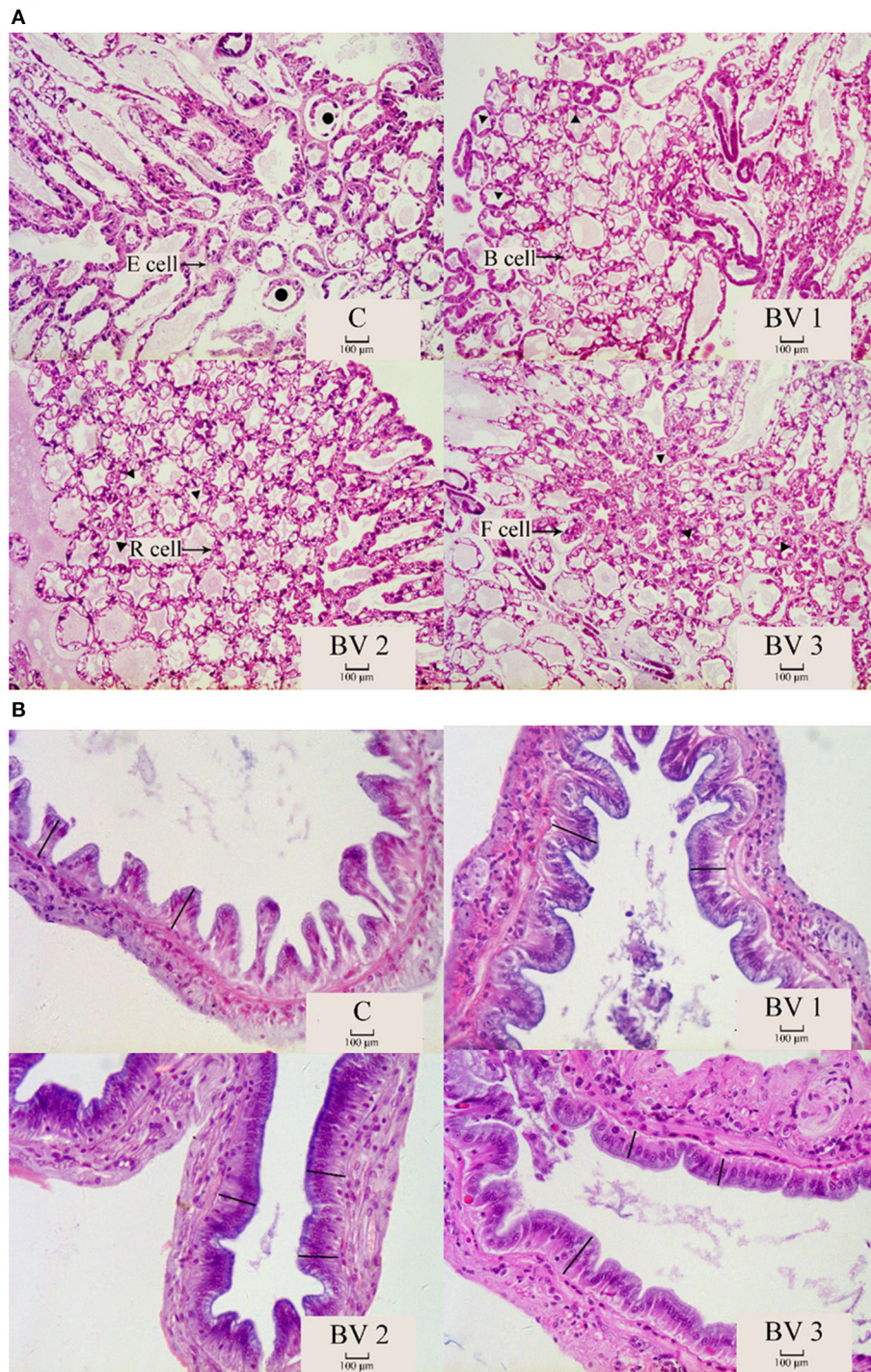


## DISCUSSION

Many strains of some *Bacillus* sp. are currently used as probiotic dietary supplements in aquatic animal feeds (Foyosal and Lisa, 2018; Kewcharoen and Srisapoome, 2019; Kuebutornye et al., 2019; Zhai et al., 2019; Zhou et al., 2019; Liu et al., 2020). These probiotics not only produce certain essential micronutrients that promote better growth and feed utilization of the hosts (Xie et al., 2019) but also participate in the digestion processes that break down nutrients, such as carbohydrates, proteins, and lipids by producing extracellular enzymes (e.g., amylase, trypsin, lipase) (Wang et al., 2007; Zhang et al., 2020). In this study, a basal diet with continuous supplementation of *B. velezensis* was shown to increase the growth performance of shrimp, which is consistent with the results of previous studies in tilapia (Galagarza et al., 2018; Hassaan et al., 2018), carp (Fan et al., 2018; Jiang et al., 2019), and grouper (Li et al., 2019b). The observed improvement in growth performance could be ascribed to the enhanced intestinal digestive enzyme activities (Bokkenheuser et al., 1988; Xu et al., 2003; Fu et al., 2018). In the present study, the digestive enzymes, such as  $\alpha$ -amylase (known to catalyze the hydrolysis of starch into sugars), lipase (known to catalyze the hydrolysis of fats and lipids), and trypsin (catalyzing the hydrolysis of proteins into smaller peptides) significantly increased in the treated groups compared with the untreated shrimp (Rawlings and Barrett, 1994; Svendsen, 2000). Similar observations were recorded in *Apostichopus japonicus* and *Salmo salar* L. that were fed diets supplemented with *B. velezensis* (Wang et al., 2018; Wang J. et al., 2019). It was also noted that *Bacillus* genus might secrete a wide range of exoenzymes that aid in the nutritional enhancement of the host. As a result, the increase in digestive enzyme activities can be linked to the elevated beneficial bacteria in the *Bacillus*-treated groups since they secrete chemical compounds, such as protease, amylases, and  $\beta$ -galactosidases. Notably, the GPT and GOT activities were reduced in the *B. velezensis*-supplemented groups, indicating that no hepatopancreas tissue damage or dysfunction was induced by the addition of probiotics (Cao et al., 2012).

The modulation of immune responses and disease resistance is a major function induced by probiotics. As a result, both cellular and humoral immunity are stimulated in response to a pathogenic challenge (Goulart et al., 2019; Ng et al., 2019; Wang G. et al., 2019). In this study, the phagocytosis of *L. vannamei* hemocytes was increased with the addition of *B. velezensis*. Similarly, Rengpipat et al. (2000) found that the use of *Bacillus* S11 could activate cellular immune defenses of *Penaeus monodon*. During phagocytosis, respiratory bursts were used to kill bacterial pathogens, relying in particular on ROS, but their overproduction can cause damage to biomolecules, such as lipids, proteins, and nucleic acids, and generate oxidative stress (Spencer et al., 2019). After infection, BVs groups significantly reduced the damage of ROS to the body, possibly by modulation of the internal antioxidants and antioxidative systems (Chen et al., 2015). The SOD and CAT are considered to be molecular biomarkers for evaluating the oxidative stress status of aquatic organisms (Valavanidis et al., 2006; Yang et al., 2015). After the bacterial challenge, the activities of SOD and CAT showed similar trends, increasing slightly at first and then decreasing,





**FIGURE 3 |** The morphological structure of hepatopancreas (A) and intestine (B). C, 0 CFU/g; BV1, 10<sup>5</sup> CFU/g group; BV2, 10<sup>7</sup> CFU/g group; BV3, 10<sup>9</sup> CFU/g group. ●: liver tubules are round; ▲: the liver tubules are star-like or polygonal. -: intestinal villus.

which suggest that dietary supplemented probiotics improved the hepatopancreas antioxidant capacity of shrimp. ACP and AKP are necessary for phosphorylation and dephosphorylation, which are very important to the crustacean immune system

(Yang et al., 2007). In the present study, the AKP activities in *B. velezensis*-supplemented groups were significantly higher than that in the control group. Similar results have also been found by Amoah and Cai (Amoah et al., 2019b; Cai et al.,



**TABLE 5** | Statistics of the morphology and structure of hepatopancreas and midgut of *L. vannamei* fed diets containing 0 CFU/g (C), 10<sup>5</sup> CFU/g (BV1), 10<sup>7</sup> CFU/g (BV2), and 10<sup>9</sup> CFU/g (BV3) *B. velezensis* for 6 weeks.

| Items                                 | Treatments               |                           |                           |                           |
|---------------------------------------|--------------------------|---------------------------|---------------------------|---------------------------|
|                                       | C                        | BV 1                      | BV 2                      | BV 3                      |
| Star-shaped or polygonal liver tubule | 52.00±8.19 <sup>b</sup>  | 93.00±14.47 <sup>a</sup>  | 100.00±15.25 <sup>a</sup> | 81.00±9.49 <sup>a</sup>   |
| Circular liver tubule                 | 10.00±2.64 <sup>a</sup>  | 7.00±2.45 <sup>ab</sup>   | 1.75±2.06 <sup>b</sup>    | 2.50±1.73 <sup>b</sup>    |
| Intestinal villi (μm)                 | 94.71±34.65 <sup>b</sup> | 130.37±27.00 <sup>a</sup> | 127.83±35.15 <sup>a</sup> | 123.80±47.36 <sup>a</sup> |

The values (mean of 8 replicates ± SD) with different letters (a, b, c) represent statistically significant differences based on the LSD method ( $P < 0.05$ , one-way ANOVA).

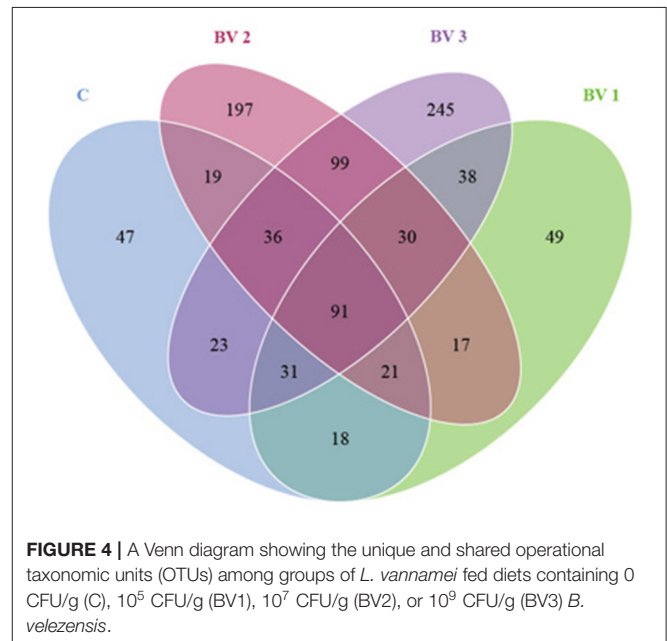
**TABLE 6** | Operational taxonomic unit (OTU) numbers, species richness, and diversity indices for the intestinal microbial community diversity analysis of *L. vannamei* fed different levels of *B. velezensis* BV007.

| Sample name      | C                          | BV1                       | BV2                        | BV3                        |
|------------------|----------------------------|---------------------------|----------------------------|----------------------------|
| OUT numbers      | 316.00±3.00 <sup>ab</sup>  | 286.33±27.51 <sup>b</sup> | 573.00±137.41 <sup>a</sup> | 587.00±74.06 <sup>a</sup>  |
| Observed_species | 273.50±12.50 <sup>ab</sup> | 237.67±29.13 <sup>b</sup> | 523.67±137.87 <sup>a</sup> | 518.00±77.01 <sup>ab</sup> |
| Shannon          | 2.74±0.01                  | 2.65±0.65                 | 4.58±1.02                  | 4.39±0.81                  |
| Simpson          | 0.69±0.031                 | 0.66±0.13                 | 0.79±0.10                  | 0.84±0.06                  |
| Chao1            | 290.91±4.74 <sup>ab</sup>  | 287.26±29.45 <sup>b</sup> | 606.81±136.92 <sup>a</sup> | 593.92±54.11 <sup>a</sup>  |
| ACE              | 302.08±9.728 <sup>ab</sup> | 300.43±24.48 <sup>b</sup> | 587.27±123.42 <sup>a</sup> | 582.96±67.67 <sup>a</sup>  |
| PD_whole_tree    | 34.70±1.07 <sup>ab</sup>   | 25.48±2.86 <sup>b</sup>   | 53.34±11.39 <sup>a</sup>   | 52.64±5.33 <sup>a</sup>    |

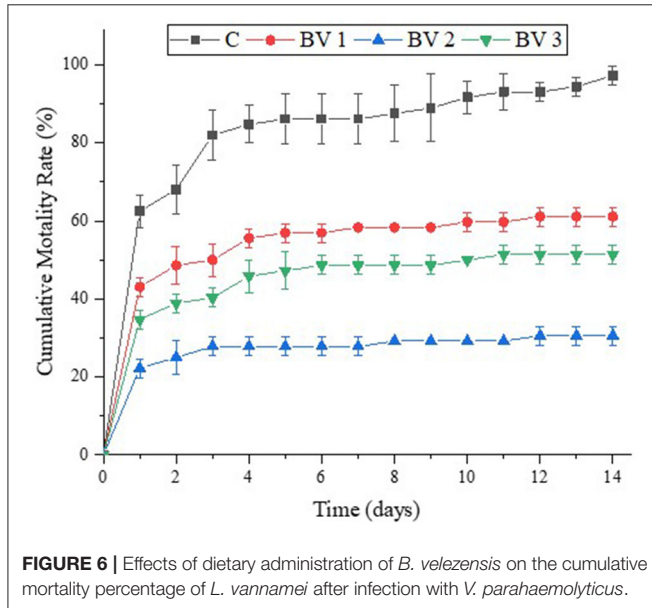
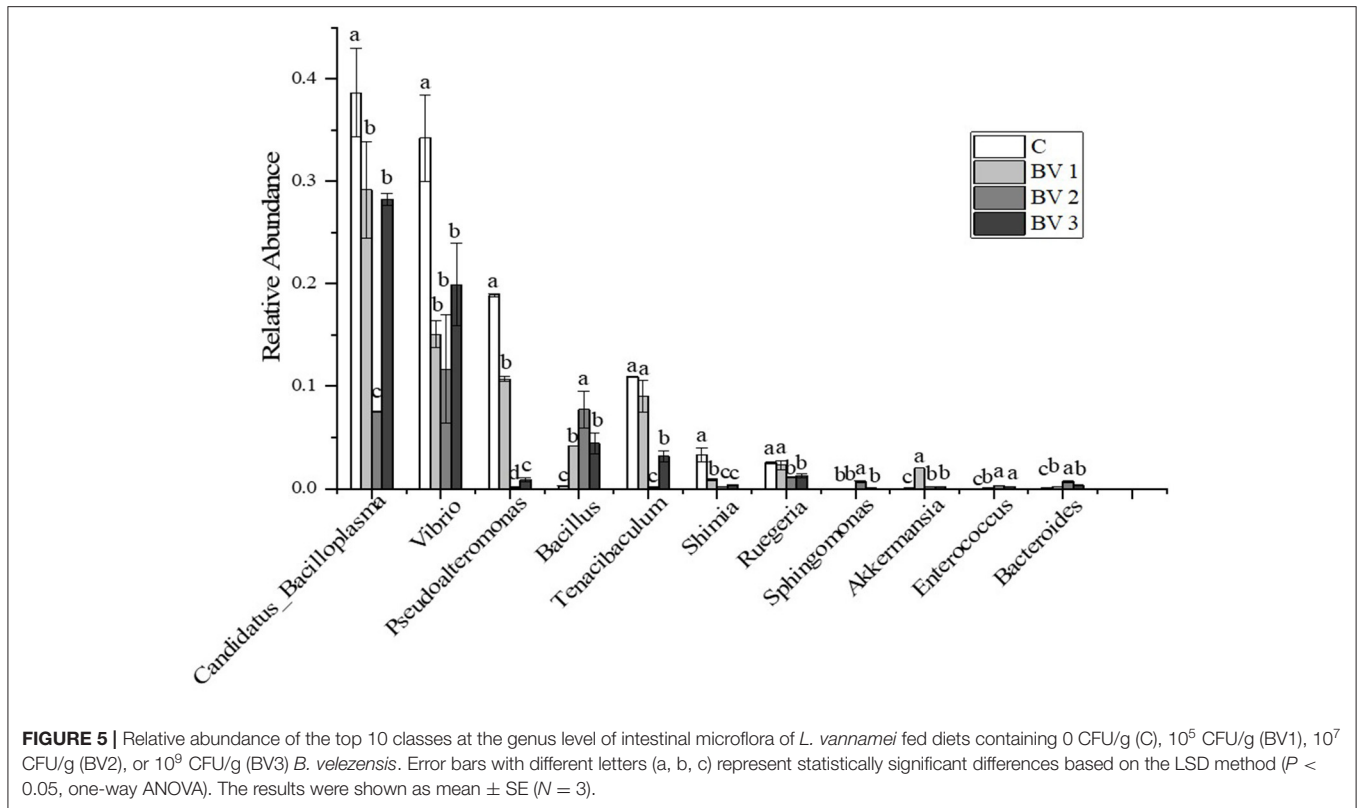
The values (mean of 3 replicates ± SE) with different letters (a, b, c) represent statistically significant differences based on the LSD method ( $P < 0.05$ , one-way ANOVA).

2019). The results suggested that dietary supplementation of *Paenibacillus polymyxa*, *B. licheniformis*, and *Bacillus flexus* enhanced the growth, hepatopancreas immune, and antioxidant activities of shrimps. In addition, the expressions of immune-related genes were highly induced in BVs groups. Among them, *crustin* and *penaeidin 3α* are vital antibacterial peptides in shrimps, powerful for the elimination of *Vibrio* (Cuthbertson et al., 2002, 2005; Muñoz et al., 2002; Arockiaraj et al., 2013). The enhanced expression of these genes contributes to resist invading bacteria and improve non-specific immunity (Muñoz et al., 2002; Arockiaraj et al., 2013; Cuthbertson et al., 2005). Overall, the dietary supplemented *B. velezensis* BV007 could boost the innate immunity of shrimp at an appropriate level.

The intestinal microbiota of shrimp plays an essential role in mediating immunity, nutrient metabolism, and energy homeostasis (Zhang et al., 2020). The previous studies have shown that probiotics in the shrimp diet could effectively modulate the intestinal microbiota, improve growth performance, and enhance disease resistance (Yang et al., 2015; Suo et al., 2017; Amoah et al., 2019a; Xie et al., 2019). In the present study, a greater diversity of bacterial species was found in the BV007 supplemented groups than that in the control group. The reason may lie in the changed metabolic activities or beneficial effects on gut microbiota regulated by *B. velezensis* (He et al., 2019; Li et al., 2019a). Notably, the *Vibrio* species are severe pathogens in aquatic organisms (Hsu and Chen, 2007). In 2013, severe acute hepatopancreatic necrosis disease (AHPND) was induced by *V. parahaemolyticus* (Tran et al., 2013) causing mass mortality of shrimps specifically in the affected Southeast Asian countries (Hsu and Chen, 2007). In this study, cumulative mortality of the probiotics treated groups was significantly lower



than the untreated group after *V. parahaemolyticus* challenge. It suggested that *B. velezensis* 007 could improve the disease resistance of *L. vannamei*. Similar results were also proved by Zokaeifar et al. (2012) and Amoah et al. (2019b). They suggested that *Bacillus subtilis* and *Bacillus coagulans* ATCC 7050 enhanced the anti-disease ability in shrimp. The results might be attributed to the increased abundances of beneficial genus bacteria *Bacillus*



and enhanced immune responses in the treated groups (Amoah et al., 2019b).

The present study showed that diet supplementation with the potential probiotic *B. velezensis* BV007 could significantly promote growth performance, enhance immune response, and

disease resistance in *L. vannamei*. Based on these findings, we conclude that a dose of  $10^7$  CFU/g feed provides health and growth benefits by improving the digestive enzyme activities, immune responses, intestinal development, and gut microflora.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

## AUTHOR CONTRIBUTIONS

SH conceived and designed the experiments. LC and CL conducted the experiments. BL, HZ, and QZ analyzed the data. LR, XZ, CS, and JG contributed reagents, materials, and analytical tools. LC, CL, and SH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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