Fish and Shellfish Immunology 87 (2019) 796–808

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Dietary supplementation of probiotic *Bacillus coagulans* ATCC 7050, improves the growth performance, intestinal morphology, microflora, immune response, and disease confrontation of Pacific white shrimp, *Litopenaeus vannamei*

Kwaku Amoah[a,c], Qin-Cheng Huang[a,c], Bei-Ping Tan[a,b,c], Shuang Zhang[a,b,c], Shu-Yan Chi[a,b,c], Qi-Hui Yang[a,b,c], Hong-Yu Liu[a,b,c], Xiao-Hui Dong[a,b,c,*]

[a] Laboratory of Aquatic Animal Nutrition and Feed, College of Fisheries, Guangdong Ocean University, Zhanjiang, Guangdong, 524088, China
[b] Key Laboratory of Aquatic, Livestock and Poultry Feed Science and Technology in South China, Ministry of Agriculture, Zhanjiang, Guangdong, 524000, China
[c] Aquatic Animals Precision Nutrition and High-Efficiency Feed Engineering Research Centre of Guangdong Province, China

ARTICLE INFO

Keywords:
Bacillus coagulans ATCC 7050
Litopenaus vannamei
Growth
Immune
Microflora

ABSTRACT

The present study assessed the effects of probiotic bacterium *Bacillus coagulans* ATCC 7050 (BC) fed at different inclusion levels (0 (BO), 1×10^6 (BC1), 1×10^7 (BC2) and 1×10^8 (BC3) CFU g^{-1} feed) on growth, feed utilization, body composition, intestinal morphology, microflora, immune response, and resistance to *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei*. After 56 days of the feeding trial, the survival rate ranged from 83.33 to 94.17% with no significant difference between dietary treatments ($P > 0.05$). Dietary probiotic supplementation also affected the intestinal microflora composition. At the phylum level, *Proteobacteria* accounted for the majority of bacteria followed by *Bacteroidetes* irrespective of the group. At the genus level, the abundance of opportunistic pathogenic bacteria, such as *Vibrio*, *Tenacibaculum*, and *Photobacterium* significantly decreased ($P < 0.05$) with an increasing probiotic concentration, and BC3 group experiencing the least. Additionally, increasing probiotic inclusion in diet downregulated the abundance of *Muricauda*, *Kangiella,* and *Shewanella* in shrimps, with the least, observed in the BC3 group. However, beneficial bacteria *Pseudoalteromonas* significantly increased ($P < 0.05$) in the intestines of shrimp fed BC3 diet ($P < 0.05$) compared to other groups including the control. Compared to the control, a significant increase ($P < 0.05$) of the probiotic treated groups in the final weight, weight gain rate (WGR), specific growth rate (SGR), condition factor (K), activity of lysozyme (LYZ), acid phosphatase (ACP), superoxide dismutase (SOD), total protein (TP), albumin (ALB) in serum, glutathione peroxidase (GSH-Px) in serum and liver, and a significant decrease ($P < 0.05$) in feed conversion ratio (FCR), triglyceride (TG) in serum, and Malondialdehyde (MDA) in serum and liver were achieved. Increasing probiotic treatment again improved the digestive ability, thus; a significant increase in the activities of lipase, amylase, trypsin, and an enhancement in the villus height, villus width, and muscle thickness of the intestines of the shrimps which correspondingly alleviated intestinal injury. Furthermore, the supplementation of probiotics in challenge test significantly ($P < 0.05$) enhanced the resistance of shrimp against *V. parahaemolyticus* infection recording BC3 to receive the highest relative percentage survival (RPS) value of 76%. In conclusion, higher inclusion levels of probiotic BC at 1×10^8 CFU g^{-1} feed (BC3) in diets can be considered to enhance the growth, intestinal morphology and microflora, immune response and resistance to *Vibrio parahaemolyticus* of *L. vannamei*.

1. Introduction

Probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [1]. Extensive studies shows that, probiotics can help improve the growth, modulation of immune system, pre-digestion of anti-nutritional factors which are found in feed, provision of energy for epithelial cells, modulation of the gut microflora and disease resistance of fish and crustaceans [2–8], thus; have been an alternate additive to the criticized and banned antibiotics [9–11]. Nevertheless, critical attention including the ability to

* Corresponding author. Laboratory of Aquatic Animal Nutrition and Feed, College of Fisheries, Guangdong Ocean University, Zhanjiang, Guangdong, 524088, China.
E-mail address: dongxiaohui2003@163.com (X.-H. Dong).

https://doi.org/10.1016/j.fsi.2019.02.029
Received 11 December 2018; Received in revised form 11 February 2019; Accepted 15 February 2019
Available online 18 February 2019
1050-4648/

be cultured under larger scale, survive until reaching the gastrointestinal tract (GIT) of the host by being able to resist acidic conditions in the stomach to exert their effect, must be paid in course of selecting probionts since unsuitable ones can negatively affect nutrient's metabolism, immunomodulation, colonization resistance, and pathogens' resistance [12,13]. Until now, though continuous search for probiotics are still underway, the two most commonly used probionts, namely; lactic acid bacteria (LAB) and *Bacillus spp.*, the latter has been noted to be a burgeoning trend for the sustenance of aquaculture development [10,14] due to their enormous advantages such as long-lasting shelf life, resistance to extreme pH, UV radiation, temperatures and the vast documentation of their beneficial effects in aquaculture [10,15].

Bacillus coagulans strain within the genus *Bacillus* is a gram-positive spore-forming bacteria, which combines both *Bacillus* and LAB properties in its actions though there is stronger stress resistance in LAB. *B. coagulans* are again noted to survive extremes of heat, the acidity of the stomach, bile salts and inhibit enteropathogens. Furthermore, they are known to improve digestive health by posing antagonistic effects on pathogens [3,16–19].

Pacific white shrimp (*Litopenaeus vannamei*) has been noted to be the most widely farmed and economically important specie in China (accounting for 73%) and the world at large due to its ability to tolerate wide range salinities and temperature, easiness to be cultured under high stocking density, easiness to be bred, faster growth, higher survival rate, attractive size, high market demand, and resistance to diseases [20,21]. However, there have been losses of about 1.7 million tonnes (MT) (worth US $ 3.3 billion) in shrimp production caused by other factors including diseases (accounting for 297,000 T of the loss) [22–24]. Like other shrimps, pacific white shrimps lack adaptive immune features and are thus dependant on innate immune responses for detection and the elimination of pathogens with which probiotic studies have revealed positive results to tackle such menace [25]. Increasing proofs have shown a close association and positive results between intestinal bacterial communities and human health [26,27]. However, knowledge regarding probiotics' effect is unclear whether shrimps' intestinal microbial community and intestinal health (morphology) are indications of host health after its supplementation as previously reported in humans and animals [28,29], hence, the need for further studies. Thus, the primary aim of the present study were to determine the effects of dietary *Bacillus coagulans* ATCC 7050 on the growth, immune response, intestinal microflora and health (morphology and digestive enzyme activity), and disease resistance of Pacific white shrimp *L. vannamei*.

2. Materials and methods

2.1. Bacterial strain

Bacillus coagulans ATCC 7050 (BC) was procured from the Guangdong Microbial Culture Center (GDMCC), Guangdong, China. Under aseptic conditions, the bacteria was streaked and revived on de Man, Rogosa, and Sharpe (MRS) (Beijing Land Bridge Tech. Co. Ltd.) agar following the company's protocol. The purity of the strain was ratified as colonies were identified on the bases of their morphological, and biochemical characteristics. Subsequently, pure clones were inoculated and used for feed preparation.

2.2. Diets formulation and preparation

Following the AOAC [30,31] method, the basal diet's ingredients used was measured to contain 41.35% crude protein and 7.67% crude lipid with fish meal, corn gluten meal and soybean meal as the primary protein source whereas the main lipid sources were fish oil, soybean oil, and soy lecithin oil (Table 1). Moisture (10.38%) was determined by oven drying at 105 °C until a constant weight was attained, crude protein (nitrogen × 6.25) by the Kjeldahl method using an Auto Kjeldahl System (8400-Autoanalyzer, FOSS), crude lipid by petroleum ether extraction (Soxhlet method), and crude ash (10.93%) by combustion of muffle furnace which involves oven incineration at 550 °C for 5 h.

Table 1
Ingredients and nutritional composition of the basal diet.

Ingredients	Composition (%)
Brown fish meal[a]	26.0
Soybean meal[a]	13.0
Peanut meal[a]	8.0
Wheat flour[a]	26.0
Corn gluten meal[a]	9.0
Shrimp shell meal[a]	6.0
Soybean oil[a]	1.0
Fish oil[a]	1.0
Soy lecithin[a]	1.0
Vitamin premix[b]	1.0
Mineral premix[c]	1.0
Choline chloride[d]	0.5
Vitamin C[a]	0.1
Ca $(H_2PO_4)_2$[d]	1.5
Carboxy methyl cellulose[e]	1.0
Microcrystalline Cellulose[e]	3.9
Total	100
Nutrient Index	**Proximate composition (%)**
Crude protein	41.35
Crude lipid	7.67
Crude ash	10.93
Moisture	10.38

[a] Ingredients purchased from Zhanjiang HaiBao Feed Factory, Zhanjiang, Guangdong, China.
[b] Vitamin premix supplied the following per kg of the diet: vitamin A, 22,500 IU; vitamin D_3, 6,000 IU; vitamin E, 200 mg; vitamin K3, 40 mg; vitamin B1, 30 mg; vitamin B2, 45 mg; vitamin B6, 35 mg; vitamin B12, 0.25 mg; calcium pantothenate, 150 mg; niacin, 225 mg; folic acid, 12.5 mg; biotin, 0.5 mg; inositol, 500 mg (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China).
[c] Mineral premix provided the following per kilogram of diet: Fe, 60 mg; Zn, 24 mg; Mn, 16 mg; Cu, 1.4 mg; Co, 0.2 mg; Se, 0.1 mg; I, 0.2 mg (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China).
[d] Obtained from Shanghai Macklin Biochemical Co. Ltd., 1288 Canggong Rd., Shanghai, China.
[e] Obtained from Shantou Xilong Chemical Factory, Guangdong, China.

In preparing the experimental diets, pure clones of BC were inoculated in a 500-ml flat bottom flask with MRS broth in a shaken incubator (180 rpm) at 37 °C for 22 h. The bacteria were harvested by centrifugation (7,000 rpm for 10 min at 4 °C) which was later washed twice with phosphate-buffered saline (PBS) at a pH of 7.2. Cell pellets were re-suspended, and the optical density (OD) measured at 600 nm [32] and found to be 0.987. Serial dilution was made, and the concentration of bacteria cells was adjusted to suit the required dosage for the various feed preparation. Instantaneously, the raw materials after visual examination for their physical appearance such as uniformity, colour and fragrance were ground and sieved using 80 mesh, stirred, intensified gradually by mixing in V-mixer type machine which was later transferred in a Hobart-type mixer where choline chloride, various lipids as well as water and bacteria with their respective doses under sterilized conditions were added to the dough by constant mixing. The four experimental diets made, namely; basal diet without probiotic, control (BO); basal diet + *B. coagulans* at 1×10^6 CFU g^{-1} feed, (BC1); basal diet + *B. coagulans* at 1×10^7 CFU g^{-1} feed, (BC2); and basal diet + *B. coagulans* at 1×10^8 CFU g^{-1} feed (BC3), were collected on steel trays, oven-dried at 60 °C for 30 min, air dried at room temperature afterwards till the moisture content reached 10% and stored at −20 °C in sealed plastic Ziploc bags until the commencement of the experiment. The viability of the BC in diet was assessed following storage of the diets at 4 and 20 °C for 8 weeks as previously reported [4,33]. Thus, 1 g amounts of feed were homogenized in 9.0 ml volumes

of saline, and serial dilutions prepared to 10^6, before 0.1 mL volumes were spread onto triplicate plates of Luria broth agar (LBA, Sangon Biotech) media. The colony counts were determined weekly after incubation for 24–48 days till the 8th week. Based on the data of the pre-experiment conducted on survival of BC which revealed the best viability to be on the first week, feeds were prepared on weekly basis to ensure high probiotic levels in the supplemented feed.

2.3. Experimental design and rearing management

Healthy juvenile batches of Pacific white shrimp, *Litopenaeus vannamei,* showing no signs of diseases (examined through the gross examination of the carapase, pods (uro, pere and pleo), gills of respective samples, thoracic and abdominal segments), no previous history of parasitic infections, and having an initial mean body weight of 0.57 ± 0.001 g were provided by the shrimp farm of the College of Fisheries, Guangdong Ocean University (Zhanjiang, Guangdong province, China). The shrimps were thus maintained in aerated cement pools (4.5 m (l) × 3.45 m (w) × 1.8 m (h)) for an acclimatization period of two weeks and hand-fed four times daily (07:00, 11:00, 17:00 and 21:00) at 10% body weight with commercial diets (purchased from Zhanjiang Aohua Feed Co. Ltd., Guangdong, China). After adaptation, a total of 480 juvenile shrimps starved 24 h, were weighed and randomly distributed into 12 fiberglass tanks (0.3 m^3) at 40 shrimps' density per tank. The juvenile shrimps were exposed to four different diets (BO, BC1, BC2, and BC3). The experiment was conducted in an indoor facility of the Marine Biological Research Base of Guangdong Ocean University under a photoperiod of natural 12 h light/12 h dark regime with a two-day interval of 50% water exchange for the first two weeks. Single-airstones provided aeration and water quality of temperature, dissolved oxygen, pH, and salinity maintained as 28–30 °C, ≥ 6 mg L^{-1}, 7.8–8.2, and 28.5–32‰ respectively.

2.4. Sampling for analysis

2.4.1. Growth performance

At the cessation of the 56-day experimental period, shrimps were starved 24 h before harvest. The total remaining shrimp number was counted, and the mean body weights of shrimps were measured. Based on the recordings, growth parameters such as survival rate (SR), specific growth rate (SGR), weight gain rate (WGR), feed conversion rate (FCR), and condition factor (K) were determined individually following the calculations of Tekinay and Davies [34]; WGR, $\% = 100 \times \frac{[\textit{Final body weight (g)} - \textit{Initial body weight (g)}]}{\textit{Initial body weight (g)}}$; SGR, $\% = 100 \times \frac{\ln[\textit{Final body weight (g)}] - \ln[\textit{Initial body weight (g)}]}{\textit{Days of experiment}}$; FCR $= \frac{\textit{Total feed intake (g)}}{[\textit{Final body weight (g)} - \textit{Initial body weight}}$; K, $\% = 100 \times \frac{\textit{Body weight (g)}}{\textit{Body length (cm)}3}$.

2.4.2. Serum and hepatopancrease samples

After the final weighing, five shrimps were randomly selected to collect blood and liver samples. The blood was collected at their ventral sinus with 1-mL sterile syringes into 1.5-mL eppendorf tubes and stored at 4 °C overnight. Stored blood samples were centrifuged (4,000 rpm for 10 min at 4 °C) and the supernatant stored at – 80 °C for subsequent serum biochemical analysis. The same shrimp samples were dissected to collect intestinal samples which were placed into eppendorf tubes to be frozen immediately with liquid nitrogen and were later stored at – 80 °C before usage. Five shrimps from each tank were again selected randomly and kept in sealed ziploc bags and stored at −20 °C for whole body composition analysis. For the histological studies, five shrimps from each tank were randomly selected to remove the intestinal samples which were kept in Bouin's fluid (pyric acid, saturated aqueous, 75.0 ml; formalin, 25.0 ml, glacial acetic acid, 5.0 ml) for future analysis.

2.5. Proximate composition analysis

Using the standard methodology of the Association of Official Analytical Chemists [31], the moisture, crude protein, crude lipid, and ash contents of the shrimp's whole body were determined as previously described (see section 2.2).

2.6. Intestinal microbiota community discovery and analysis

The intestinal community of the 16S sequencing was performed according to Ref. [35] with slight changes. Briefly, total microbial DNA was extracted from stool samples using the E.Z.N.A. ™ stool DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer's protocols. The 16S rDNA V3—V4 region of the gene were amplified by PCR (95 °C for 2 min, followed by 27 cycles at 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 30 s and a final extension at 68 °C for 10 min) using primers 341F:CCTACGGGNGGCWGCAG; 806R:GGACTACHVGGGTATCTAAT, where the barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 50 μL mixture containing 5 μL of 10 × KOD Buffer, 5 μL of 2.5 mM dNTPs, 1.5 μL of each primer (5 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA. High-throughput sequencing was performed using Illumina Hiseq 2500 sequencing. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina platform according to the standard protocols. The raw reads were deposited into NCBI Sequence Read Archive (SRA) database (Accession Number: SRP170653).

To obtain high-quality clean reads, raw reads were further filtered by removing reads containing more than 10% of unknown nucleotides (N) and those containing less than 80% of bases with quality (Q-value) > 20. Paired and clean reads were merged as raw tags using FLASH (v 1.2.11) [36]. The reads were demultiplexed, quality-filtered and processed using QIIME (v 1.9.1) [37]. The effective tags were clustered into operational taxonomic units (OTUs) of ≥ 97% similarity using UPARSE [38] where the tag sequence with the highest abundance was selected as a reprehensive sequence within each cluster. Taxonomic richness estimators and community diversity were determined for each library in Mothur (version 1.39.1, http://www.mothur.org/). Alpha diversity indexes, including Chao 1, ACE, Shannon [39] and Simpson [40] were used for assessing community diversity. Unique species among groups were shown by Venn analysis whereas normalized abundance was shown by heatmap using pheatmap.

2.7. Biochemical measurements

Hepatic samples weighed and homogenized in a 0.9% saline at a ratio of 1:9 (hepatic tissue: saline) using a bead homogenizer in ice for 10 min were centrifuged (3,500 rpm for 10 min at 4 °C) and the supernatant collected and stored at −80 °C for subsequent liver enzyme activities' analysis.

The application of the biuret method [41] and bromocresol green calorimetry technique [42] were used to determine the serum total protein (TP) and albumin (ALB) respectively. Globulin protein (GLO) content was obtained by subtracting the ALB from the TP. Following the turbidimetric method, the serum lysozyme (LYZ) activity was determined as described by Ellis [43] with some slight modifications. Briefly, 100 μl of serum was added to 1 ml lyophilized *Micrococcus lysodeikticus* (cultured in LB media (Sangon Biotech) at 37 °C, centrifuged at 6,000 rpm at 4 °C for 5 min, washed twice, re-suspended in PBS) adjusted to a concentration of 0.243 mg ml^{-1} (c/v) in PBS, pH 6.4. The OD was recorded at 530 nm at 1 and 20 min at 22 °C with one unit of lysozyme activity being defined as the amount of serum that caused a decrease in the OD of 0.001 units/min which was later expressed as U/

ml. Triglyceride (TG) content was measured using a commercial kit (provided by Nanjing Jiancheng Biological Engineering Institute, China) according to the manufacturer's instructions.

Serum superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px), acid phosphatase (ACP) and malondialdehyde (MDA) were determined by commercial kits (provided by Nanjing Jiancheng Biological Engineering Institute, China) following the company's protocol. The SOD activity was determined based on its ability to inhibit the photochemical reduction of nitro blue tetrazolium and the colour formed at the end of the reaction could be extracted into butanol and measured at 550 nm. GSH was estimated based on the reaction ability of ditho-dinitrobenzoic acid with sulfhydryl compounds to produce relatively stable yellow colour at 405 nm absorption peak. GSH is determined based on the concentration of the yellow compounds. GSH-Px is preferably represented by catalyzed GSH reaction rate by measuring absorbance at 412 nm. ACP in serum was spectrophotometrically measured using disodium phenyl phosphate as a substrate with an acid phosphatase detection kit (Nanjing Jiancheng, Bioengineering Institute, China) measured at 530 nm. MDA was examined by the thiobarbituric acid (TBA) technique in the glacial acetic medium. Lipid hydroperoxide decomposition products can condensate with TBA to produce red compounds at 532 nm absorbance peak.

2.8. Evaluation of intestinal enzyme activity

The intestinal samples of the shrimps were weighed, and homogenized in 0.9% aseptic saline at a ratio of 1:9 (tissue: saline) using a bead homogenizer in ice for 10 min of which the homogenate was centrifuged (2,500 rpm for 10 min at 4 °C) and the supernatant collected in a 1.5-mL eppendorf tubes for the digestive enzyme activity analysis. The intestinal protein concentration was determined as described previously (see section 2.7).

Amylase (AMS), trypsin (TRP), and lipase (LPS) activity were determined by the colorimetric method using commercial kits (Nanjing Jiancheng, Bioengineering Institute, China) and the OD read in a spectrophotometer at 660, 253, and 540 nm wavelength respectively.

2.9. Histological structure of intestine

Following fixation, the gut segments removed from bouin's fluid were dehydrated with different gradients of ethanol concentration, cleaned in toluene and embedded in paraffin to make solid wax blocks. By using a rotary microtome, these solid wax blocks were then cut as continuous section blocks into 5 μm sections with the segmented tissues being stained with hematoxylin-eosin (H&E). They were thus examined on a microscope (Olympus, model BX51, Serial number: 9K18395, Tokyo, Japan) and the villus height (VH), villus width (VW) and intestinal epithelial muscle thickness (MT) were measured using the software Image-Pro Plus 6.3 (Media Cybernetics, Inc., Rockville, USA) following the procedure of Bullerwell et al. [44]. By entirely viewing from tips of villus to the submucosa, with no ruined edges, the villi were determined. The VW was also measured at the midpoint of each villus. The intestinal MT was measured from the inner edge of the muscularis mucosae to the outer edge of the serosa. As many villi as possible were measured, up to 10 villi per slide and not less than five. If more than 10 were able to be measured, the villi were chosen to be as evenly spaced around the intestine sample as possible. Slides with fewer than six suitable villi per slide were excluded.

2.10. Assessment of the protective effect of probiotic on Vibrio parahaemolyticus

Vibrio parahaemolyticus, provided by MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou, P. R. China, was used for the challenge study on each group. *V. parahaemolyticus* was cultured in 100 ml Luria broth (LB media, Sangon Biotech) in a 250 ml flat bottom flask which was kept in a shaken incubator (180 rpm at 37 °C for 20 h) with the cells pellets, obtained through centrifugation (7,000 rpm for 10 m at 4 °C). Supernatant were removed and the bacterial cells re-suspended in PBS and serially diluted to obtain graded doses (10^6, 10^7, 10^8 and 10^9 CFU/ml) of *V. parahaemolyticus* which was used to conduct a prior experiment lasting 14 days. Mortalities were monitored daily to obtain a suitable concentration of *V. parahaemolyticus*. The 14-day lethal dose 50 (LD_{50}) determined by the intramuscular injection of the graded doses of *V. parahaemolyticus* into 40 shrimps of the same sizes as the final experimental shrimp size, calculated following Reed and Muench [45] was 1.0×10^8 CFU/ml/shrimp as evaluated by the preliminary experiment. At the end of the 56-day feeding trial, 10 shrimps from each replicate were randomly selected and intramuscularly injected with 0.2 ml of the suspended bacteria (1.0×10^8 CFU/ml) at the third abdominal segment for the disease resistance test. For negative control, a group of 30 shrimps were injected with PBS, and the mortality in each group recorded up to the 14th day of the challenge. The physiological changes were observed daily, and the dead shrimp removed regularly. Cumulative mortality (%) and relative percent survival (RPS %) were calculated following the formula of Liu et al. [46].

2.11. Data statistics

All data were subjected to one-way analysis of variance (ANOVA) using statistical package for social sciences (SPSS) software for windows (IBM SPSS v20.0, Inc., 2010, Chicago, USA). Differences between means were tested by the Tukey's HSD test. A difference was considered to be statistically significant ($P < 0.05$) and the results presented as mean ± S.E. (standard error).

3. Results

3.1. Growth performance, feed utilization, survival, and whole body composition

Growth performance parameters were shown in Table 2. There were no differences in SR among treatments ($P > 0.05$). Increasing BC inclusion in the diet significantly affected the growth performance and feed utilization ($P < 0.05$). The shrimp in the BC2 and BC3 groups showed significantly higher final weight (FW), WGR and SGR than those in the BC1 and the BO group. However, between the BC2 and BC3 groups, there were no significant differences ($P > 0.05$) observed in the FG, WGR, and SGR. The lowest FCR was recorded from the BC3 group, followed by BC2, BC1, and BO.

After the 56-days feeding trial, the results of proximate whole body composition except moisture were found to be significantly ($P < 0.05$) higher with the probiotic-treated diet with better elevation being observed in BC3 group, followed by BC2 and BC1 when compared to the control. The reverse was recorded in the case of the moisture content (Table 2).

3.2. Microbiota of the gut analysis

3.2.1. Microbiota of the gut richness and diversity analysis

The topmost abundant OTUs at the genus level as inferred by GraPhlAn are shown in the cladogram of intestinal microbiota, with *Proteobacteria* being the most abundant (Fig. 1). The OTUs and Alpha diversity statistics of the intestinal microbiota in *L. vanamei* are presented in Table 3. Significantly, higher OTUs were observed in the intestines of shrimp group fed BC3 diets ($P < 0.05$), and the lowest in the BO group ($P < 0.05$). Moreover, a total of 307 OTUs were shared by the four groups, and the number of unique OTUs in the BC3 group was the highest (Fig. 2). The BC3 group had a significantly higher chao1 and ACE indices than the other treated groups ($P < 0.05$) with the least being BO. The Shannon and Simpson indices ranged from 4.27 to 5.39

Table 2
Effects of different supplementation levels of BC on the growth performance and body composition of *L. vannamei*.

Parameters	BO (Control)	BC1	BC2	BC3
Growth index				
IBW (g)	0.57 ± 0.001	0.57 ± 0.001	0.57 ± 0.001	0.57 ± 0.001
FBW (g)	6.77 ± 0.08^{a}	7.29 ± 0.14^{ab}	7.50 ± 0.16^{b}	7.30 ± 0.04^{b}
WGR (%)	1088.16 ± 14.42^{a}	1178.88 ± 25.33^{ab}	1215.05 ± 27.43^{b}	1182.00 ± 5.02^{b}
SGR (%/day)	4.42 ± 0.02^{a}	4.55 ± 0.04^{b}	4.60 ± 0.04^{b}	4.56 ± 0.01^{b}
FCR	2.68 ± 0.14^{b}	2.24 ± 0.09^{a}	2.17 ± 0.03^{a}	2.15 ± 0.02^{a}
SR (%)	83.33 ± 3.00	94.17 ± 3.00	93.33 ± 3.63	93.33 ± 2.20
K (%)	0.86 ± 0.01^{a}	1.31 ± 0.05^{b}	1.27 ± 0.02^{b}	1.39 ± 0.04^{b}
Body composition (DM)				
Moisture (%)	76.44 ± 0.47^{b}	72.81 ± 0.60^{a}	71.98 ± 0.68^{a}	73.34 ± 0.33^{a}
Crude protein (%)	71.18 ± 1.7^{a}	75.56 ± 0.45^{b}	75.94 ± 0.54^{b}	76.65 ± 0.28^{b}
Crude lipid (%)	6.93 ± 0.06^{a}	7.21 ± 0.04^{ab}	7.52 ± 0.08^{bc}	7.62 ± 0.12^{c}
Ash (%)	13.10 ± 0.02^{a}	14.05 ± 0.06^{b}	15.00 ± 0.11^{c}	14.26 ± 0.08^{b}

Note: Data are mean values of three replicates ± S.E. Means in the same row without superscripts do not differ significantly ($P > 0.05$) on the basis of Tukey's HSD test.
Where; IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; K, condition factor; SR, survival rate.

and 0.86 to 0.95 respectively, with no significant differences ($P > 0.05$) being observed between groups (Table 3).

3.2.2. Microbiota composition, relative abundance analysis, and comparison

L. vannamei's heatmap analysis of intestinal bacterial abundance at the genus level showed that *Vibrio*, *Tenacibaculum*, and *Photobacterium* were more abundant in the control group than the probiotic treated groups (Fig. 3). The abundance of *Pseudoalteromonas* was seen to be elevated in the treated group with the highly significant ($P < 0.05$) going for the BC3 group.

Relative to all the groups at the phylum level, *Proteobacteria* and *Bacteriodetes* were the most abundant phyla. Contrast to the abundance of *Bacteriodetes*, the abundance of *Proteobacteria* was significantly higher ($P < 0.05$) in the BC3 and BC2 groups than as observed in BC1 and BO (Fig. 4: A).

At the family level, the abundance of *Flavobacteriaceae* was followed by *Rhodobacteraceae*, *Vibrionaceae,* and *Pseudoalteromonadaceae*. Among the families observed, the abundance of *Flavobacteriaceae* and *Vibrionaceae* increased significantly ($P < 0.05$) in the BO and BC1 group. Moreover, *Alcanivoracaceae* and *Moraxellaceae* increased significantly ($P < 0.05$) in the control group than the entire treated groups. Nonetheless, contrast to the above findings, a significantly higher ($P < 0.05$) relative abundance of *Pseudoalteromonadaceae* was observed in the treated groups compared to the untreated, with BC3 experiencing the highest (Fig. 4: B).

The microflora composition at the genus level in the intestine of *L. vannamei* is represented in Fig. 5. Regardless of the type of diet, *Vibrio* (5.24–11.92%) was the most relative abundant genus followed by *Reugeria* (2.32–10.84%) in the intestines of the shrimp. Compared to the genus abundance in the intestines of the shrimps, a significantly lower abundance of *Vibrio, Photobacterium* and *Tenabaculum* were detected in shrimps fed the treated diet compared to those fed untreated diet with the least being observed in those fed BC3 diet ($P < 0.05$) whereas

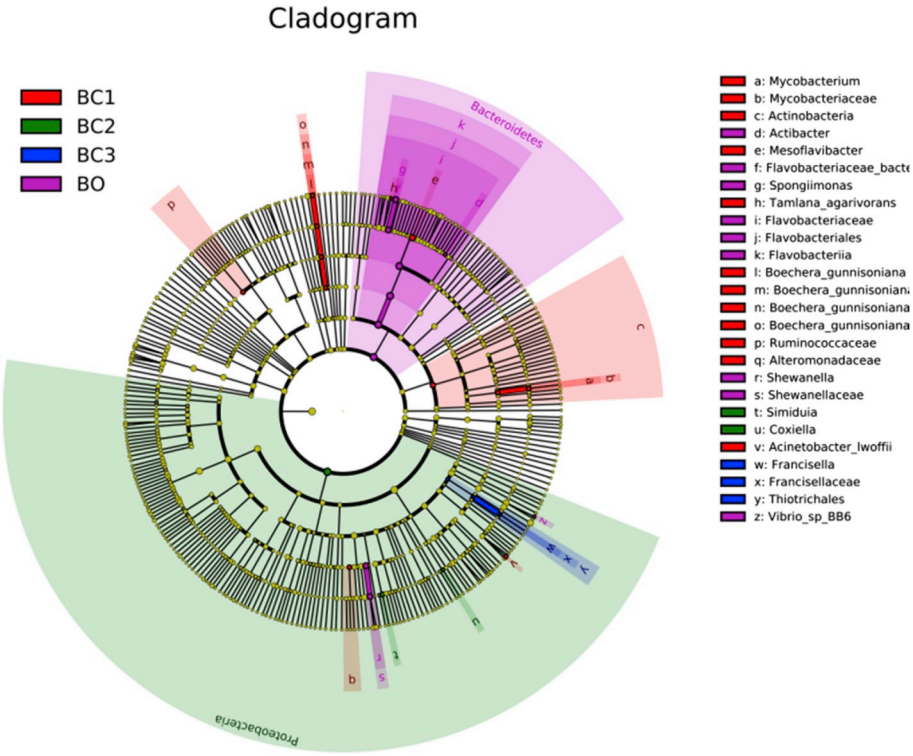

Fig. 1. Cladogram of the intestinal microbiota that are the topmost abundant as inferred by GraPhlAn at the genus level. Node size is proportional to the average abundance; colour indicates the relative concentration of the clusters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
OTU, diversity index and estimated OTU richness for the intestinal bacterial diversity analysis of *L. vannamei* at different levels of BC concentration.

Index	BO (Control)	BC1	BC2	BC3
Reads				
Raw Reads	86148	102218	123914	215669
Valid Reads	83777	99323	120387	206643
Tags	80399	95133	113882	193076
OTUs	295.5 ± 17.50^{a}	492 ± 22.00^{b}	488 ± 5.21^{b}	599 ± 3.06^{c}
Richness estimate				
Chao 1	456.17 ± 31.01^{a}	572.44 ± 10.01^{b}	661.26 ± 18.71^{b}	769.41 ± 17.26^{c}
Ace	482.29 ± 41.77^{a}	573.44 ± 12.73^{ab}	649.61 ± 4.98^{bc}	747.00 ± 10.10^{c}
Diversity estimators				
Shannon	5.39 ± 0.03	5.00 ± 0.45	4.27 ± 0.44	4.52 ± 0.11
Simpson	0.95 ± 0.00	0.89 ± 0.03	0.82 ± 0.08	0.86 ± 0.10

Note: Data are mean values of three replicates ± S.E. Means in the same row without superscripts do not differ significantly ($P > 0.05$) on the basis of Tukey's HSD test.

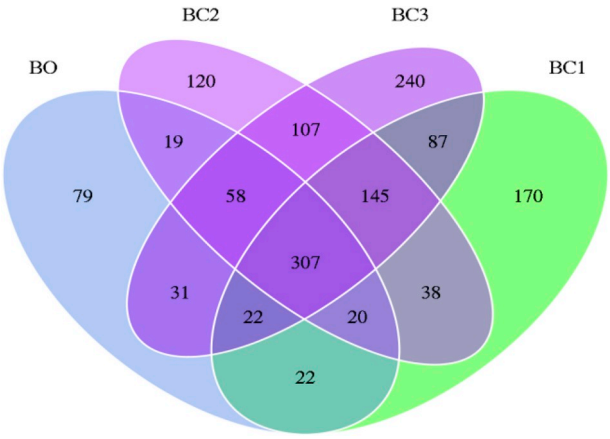

Fig. 2. Venn diagram demonstrating the distribution of OTUs shared by *L. vannamei* in the four different BC concentrations.

Reugeria genus experienced no significant difference ($P > 0.05$) among all groups. In contrast, the abundance of *Pseudoalteromonas* was highly significant in the intestines of shrimp fed the BC3 diet ($P < 0.05$) compared to the other groups including the control though there were high elevations in the abundance of the remaining treated diets.

3.3. Biochemical measurements in the serum and hepatopancrease

Biochemical indexes in serum and liver are shown in Table 4. GLO contents, unlike TP and ALB content which showed a higher significant increase ($P < 0.05$) in the treated group than the untreated group, showed no significant difference ($P > 0.05$) in all groups (Fig. 6). TG in the serum showed a decreasing trend with an increasing probiotic supplementation. TG content in serum of BC3 group experienced a significantly lower ($P < 0.05$) content than that of other groups. Serum LYZ activity was significantly higher ($P < 0.05$) in the treated group compared to the control. The serum ACP level in BC2 and BC3 rather increased significantly ($P < 0.05$) as compared to other groups. However, serum ACP in the BC1 and BO group had no significant difference ($P > 0.05$). The treated groups had a significantly higher ($P < 0.05$) SOD activity in the serum than the untreated. There were also no significant difference ($P > 0.05$) observed in the SOD activity between the BC3, BC2, and BC1 groups.

GSH levels in serum and liver had no significant difference in all groups ($P > 0.05$) although the treated groups experienced higher elevations. The GSH-Px activity in the serum was lower than that of liver (Table 4). Statistically, a higher significant difference ($P < 0.05$) of GSH-Px activity was observed in the BC3 group in both serum and liver samples than that of other groups. Shrimps fed the BC treated diet were observed to have significantly lower ($P < 0.05$) MDA contents in both serum and liver samples with the lowest value being observed in the BC3 group.

3.4. The histological study of the intestine

Fig. 7 shows the (A) photomicrographs and (B) histological measurements of the intestinal tract cross-cutting of *L. vannamei*. The epithelial cells in the intestine of shrimps in the treated group were observed to be closely arranged composing of a clear gap. They also showed tall villus tissues which also had a close integration with the basement membrane (Fig. 7A - BC2 (D1, a) and BC3 (D2)). There were some slight detachment and distortions of intestinal epithelial cells from the basement in BC1 group as compared to the groups BC2 and BC3 (Fig. 7A – BC1 (C)). There was complete separation from the basement membrane with the intestinal villus in the BO group which also experienced shorter intestinal villus compared to the other groups (Fig. 7A – BO (A)). Correspondingly, the total VH and VW tended to decrease significantly ($P < 0.05$) in the untreated group compared to the treated. Nevertheless, there were no significant difference ($P > 0.05$) found in all groups concerning the MT of the intestine of the shrimps (Fig. 7B).

3.5. Digestive enzyme activities in the gut

The results of AMS, LPS and TRP activities are shown in Fig. 8. Significantly, higher TRP activity in the intestine was detected ($P < 0.05$) in all the treated groups compared to the untreated. Moreso, the AMS and LPS activities were significantly higher ($P < 0.05$) in the BC3 and BC2 treated group than those in the BC1 and BO. Nevertheless, there were no significant differences observed in the BC1 and BO group ($P > 0.05$) concerning the AMS and LPS activity.

3.6. Challenge test

After the 14-day challenge with *V. parahaemolyticus*, the cumulative mortality rates of *L. vannamei* were shown in Fig. 9. It was observed that, the cumulative mortality was significantly lower ($P < 0.05$) in the treated groups than in the untreated, that is, 83.3%, 43.3%, 36.7% and 20% for shrimps fed with the BO, BC1, BC2, and BC3 respectively. The relative percent survival (RPS %) was highest in BC3 (76%), followed by BC2 (56%), and BC1 (48%) groups.

4. Discussion

Plethora of studies have established the beneficial effects probiotics' supplementation exerts on aquatic animals [2,12,24]. The significance of these probionts in shrimp aquaculture have been recently reviewed by Farzanfar [47] and Buruiană et al. [48]. Hitherto, the research works available have not paid much attention in revealing the relations these

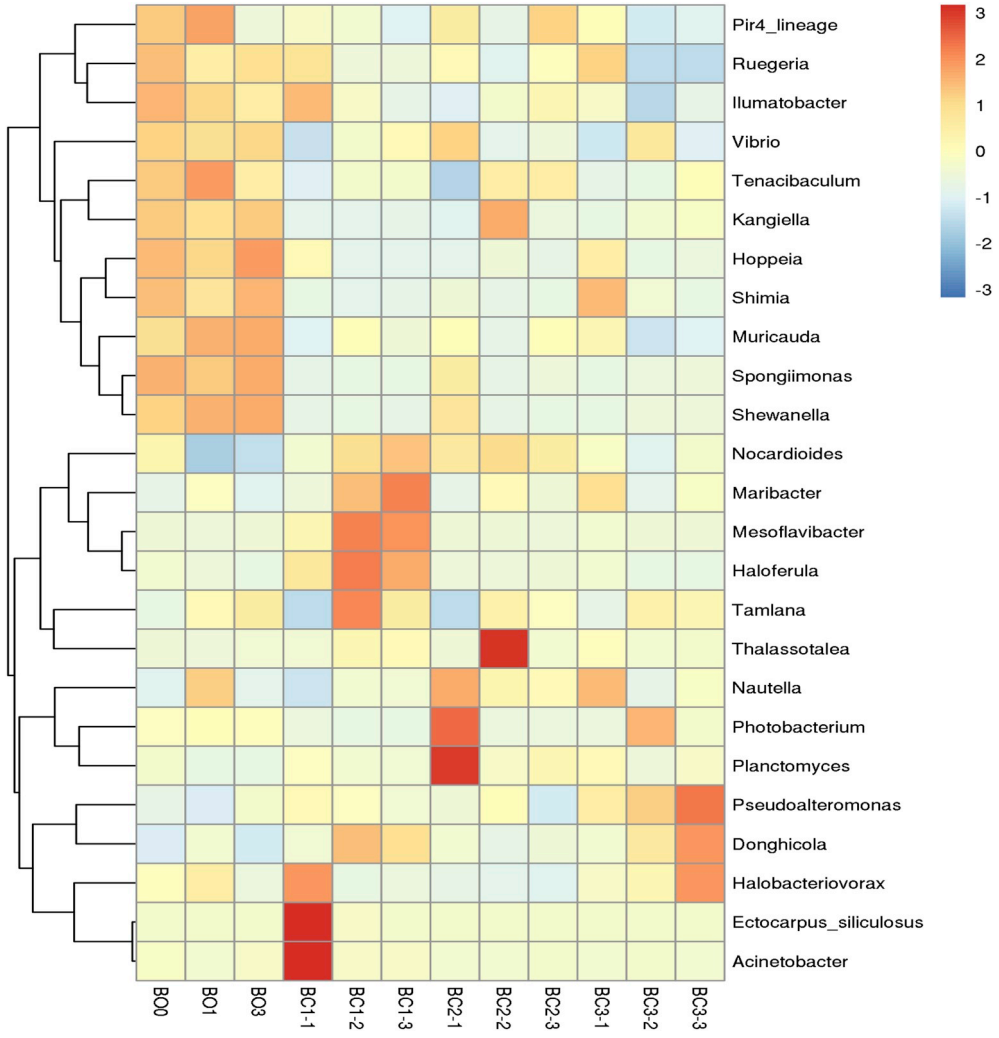

Fig. 3. Heatmap of the abundance of *L. vannamei* intestinal bacteria at the genus level at different probiotics concentrations. Phylogenetic positions are projected by the OTUs, and the taxa of OTUs are listed on the right. Colour intensity indicates the relative enrichment of OTUs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

beneficial microbes have with the gut microflora of crustaceans. In this study, the use of varied concentrations of the potential probiotic viz. BC, in *L. vannamei* as feed supplement was evaluated on the bases of its impact on the growth, intestinal microflora and health (morphology and digestive enzyme activity), immune response and disease resistance.

After the 56 day feeding trial, the results demonstrated a significant increase in the FW, WGR, SGR, K, and a significant decrease in FCR

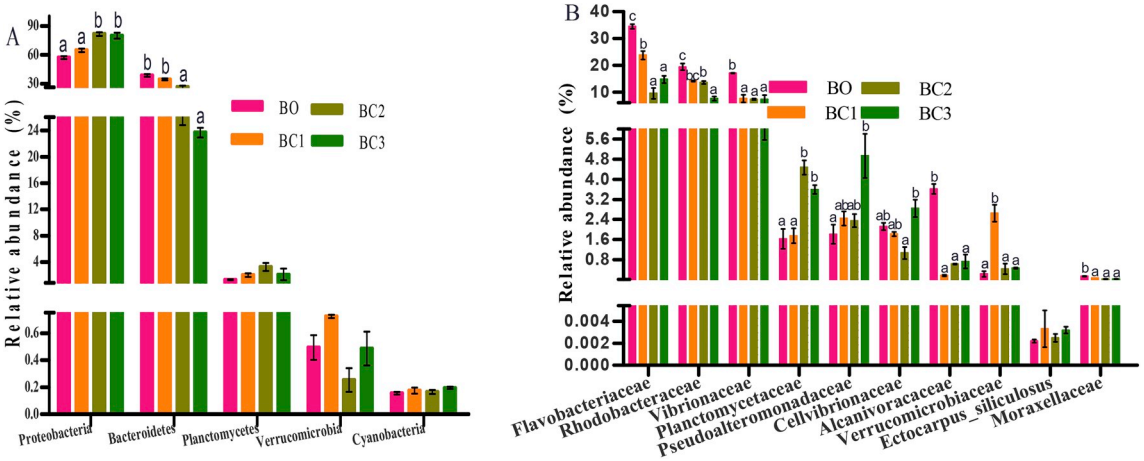

Fig. 4. Comparisons of the relative abundance of major bacteria in *L. vannamei* at the A) Phylum and B) Family level. Vertical bars represented the mean ± S.E. (Tukey's HSD, $P < 0.05$; $n = 3$). Data marked without letters do not differ significantly ($P > 0.05$) among groups.

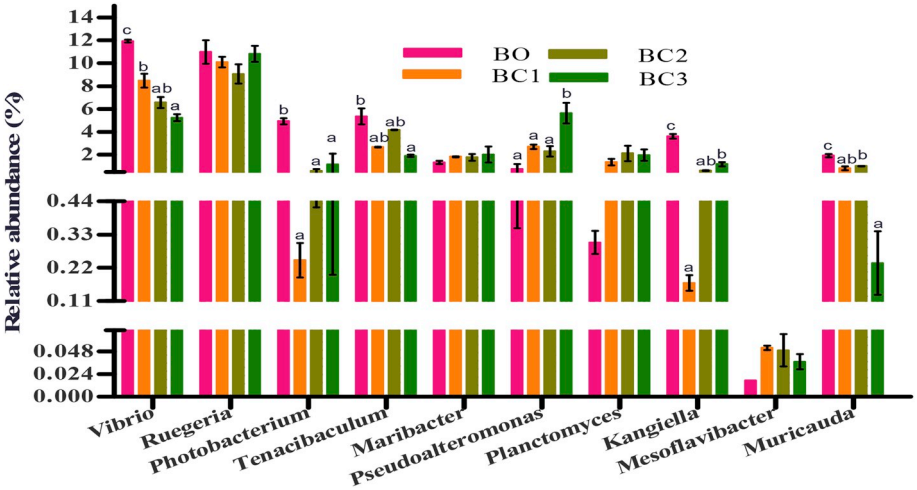

Fig. 5. Comparisons of the relative abundance of major bacteria in *L. vannamei* at the genus level. Vertical bars represented the mean ± S.E. (Tukey's HSD, $P < 0.05$; $n = 3$). Data marked without letters do not differ significantly ($P > 0.05$) among groups.

with the probiotic treated groups compared to the control. However, the highest values in FW, WGR, and lowest FCR were observed in both BC2 and BC3 group indicating that, higher doses of BC can improve the growth performance and feed utilization of *L. vannamei*. The nutritive values as reported by Vijayavel and Balasubramanian [49] is highly dependent on their biochemical constituents such as crude protein, crude lipid, ash content, moisture which also is noted to be an indication of improved meat quality. This study, contrast to moisture content, revealed a significant increase in crude protein, crude lipid and ash content in the probiotic treated group compared to the untreated. Similar significant improvement in *Litopenaeus vannamei* [7,50–52], *Fenneropenaeus indicus* [53], *Macrobrachium rosenbergii* [54], and *Penaeus monodon* [55] have been reported. Nonetheless, in elucidating the exact mechanisms of action which led to the above results, an additional study such as the examining of the intestinal microbiota which play an ardent role in the digestive enzymes activities and the intestinal health was conducted.

The intestinal microflora has recently gained much attention due to the crucial role it plays in shaping the structure. They are reported to aid the digestive functional activities which in turn improve the immune system by forming defensive barriers to protect host organisms against pathogenic invasions [56–58]. Notably, probiotic supplementations have been confirmed to alter the gut microflora of organisms by mainly producing antimicrobial agents to override the growth of other microorganisms [59–61] or vying for receptors and binding

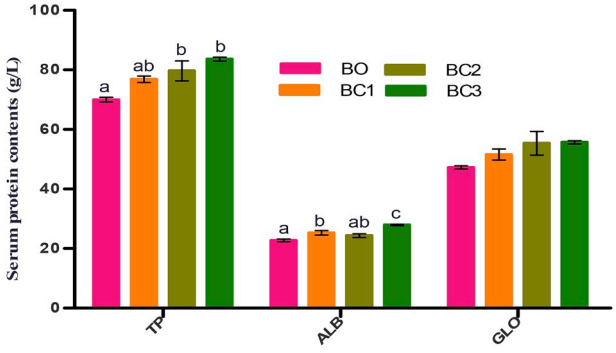

Fig. 6. Effects of different supplementation levels of BC on serum total protein (TP), albumin (ALB) and globulin (GLO) contents of *L. vannamei*. Vertical bars represented the mean ± S.E. (Tukey's HSD, $P < 0.05$; $n = 3$). Data marked without letters do not differ significantly ($P > 0.05$) among groups.

sites with host microbes [62]. In this study, BC supplementation significantly shaped the diversity of the intestinal microflora in the gut. With regardless of the type of diet, *Proteobacteria* accounted for the majority of bacteria with the second major being *Bacteriodetes* in the entire group. Correspondingly, similar results have been reported in *L. vannamei* [35,63] suggesting how adapted these are in the shrimp's gut.

Several species of the genus *Vibrio*, *Tenacibaculum,* and

Table 4
Effects of different supplementation levels of BC on immune and antioxidant factors in serum and liver of *L. vannamei*.

Parameters	BO (Control)	BC1	BC2	BC3
Serum				
TG (mmol/L)	0.55 ± 0.05^{b}	0.54 ± 0.03^{b}	0.52 ± 0.04^{b}	0.36 ± 0.00^{a}
LYZ (μg/mL)	10.33 ± 0.67^{a}	15.33 ± 0.67^{b}	15.00 ± 0.58^{b}	15.33 ± 0.33^{b}
ACP (U/100 ml)	11.65 ± 0.22^{a}	10.95 ± 0.30^{a}	21.55 ± 0.90^{b}	20.65 ± 2.37^{b}
GSH (μmol/L)	16.88 ± 0.83	17.15 ± 0.78	18.42 ± 0.34	18.36 ± 0.50
GSH-Px (U/ml)	70.36 ± 1.31^{a}	88.84 ± 1.10^{b}	85.03 ± 1.59^{a}	91.46 ± 1.46^{b}
SOD (U/ml)	718.80 ± 15.9^{a}	1088.72 ± 33.49^{b}	923.31 ± 51.39^{b}	1055.64 ± 37.56^{b}
MDA (nmol/ml)	5.56 ± 0.44^{c}	3.82 ± 0.31^{b}	2.80 ± 0.13^{ab}	2.22 ± 0.12^{a}
Liver				
GSH (μmol/L)	17.64 ± 1.15	18.38 ± 1.27	20.38 ± 0.88	22.39 ± 1.27
GSH-Px (U/mgprot)	244.26 ± 7.66^{a}	335.78 ± 11.52^{b}	361.21 ± 10.87^{b}	493.38 ± 13.39^{c}
MDA (nmol/mgprot)	5.13 ± 0.07^{d}	2.20 ± 0.09^{c}	1.73 ± 0.04^{b}	1.44 ± 0.00^{a}

Note: Data are mean values of three replicates ± SEM. Means in the same row without superscripts do not differ significantly ($P > 0.05$) on the basis of Tukey's HSD test.

Where; TG, triglyceride; LYZ, Lysozyme; ACP, Acid phosphatase; SOD, superoxide dismutase; GSH, glutathione; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

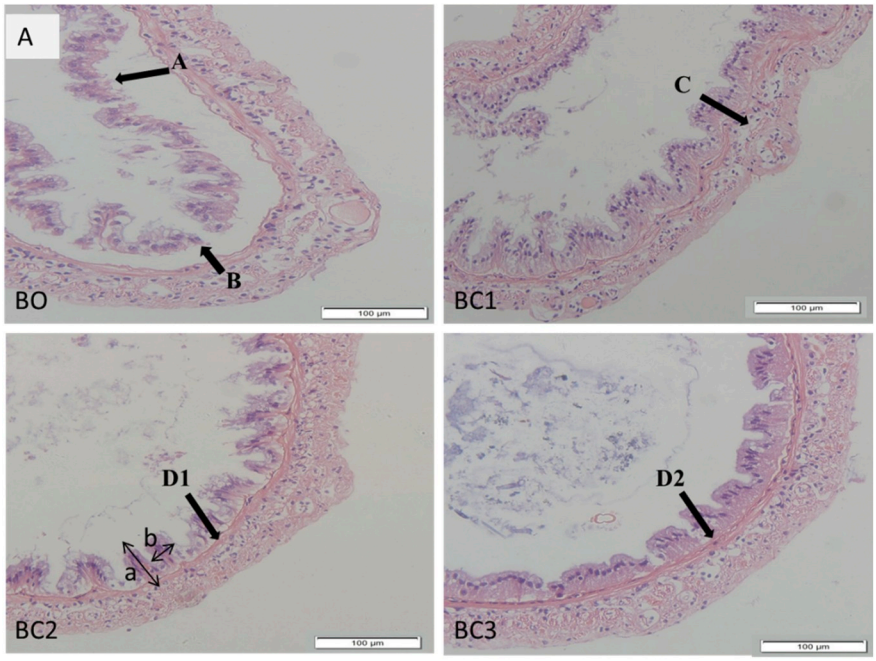

Fig. 7. Photomicrographs **(A),** and Histological measurements **(B)** of the intestinal tract cross-cutting of *L. vannamei*. **(A)** For histological comparison, arrows show the pathological changes. Magnification was 200 × , and the scale represents 100 μm. BO: arrow A and B shows the intestinal epithelial cells completely detached from the basement membrane; BC1: C shows some slight detachment of intestinal epithelial cells from the basement; BC2: D1 shows the close integration of the intestinal epithelial cells and the basement membrane, a shows the villus height, b shows the villus width; BC3: D2 shows the close integration of the intestinal epithelial cells and the basement membrane. **(B)** Vertical bars represented the mean ± S.E. (Tukey's HSD, $P < 0.05$; $n = 3$). Data marked without letters do not differ significantly ($P > 0.05$) among groups.

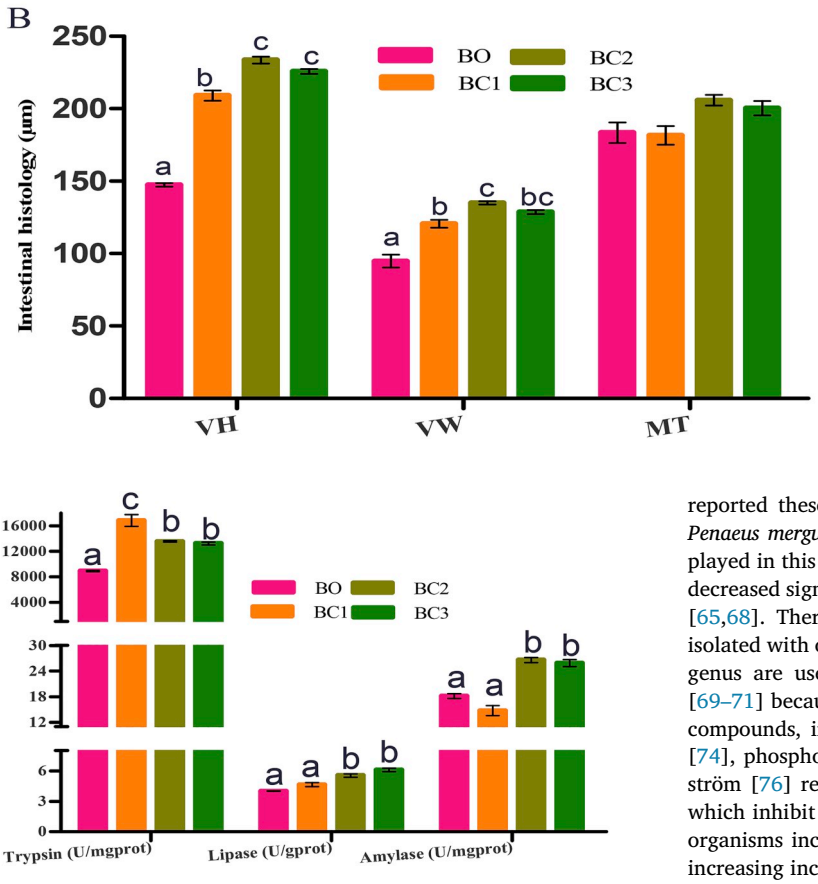

Fig. 8. Effects of different supplementation levels of BC on the intestinal digestive enzyme activities of *L. vannamei*. Vertical bars represented the mean ± S.E. (Tukey's HSD, $P < 0.05$; $n = 3$). Data marked without letters do not differ significantly ($P > 0.05$) among groups.

Photobacterium, found to be mostly abundant at the genus level in this study, are known to be opportunistic pathogens which can damage the intestinal morphology and immune mechanism. Similarly, others have reported these bacteria to be mostly abundant in *L. vannamei* [64], *Penaeus merguiensis* [65], *Penaeus monodon* [66] and others [67]. Displayed in this study, *Vibrio*, *Tenacibaculum,* and *Photobacterium* genuses decreased significantly at an increasing inclusion of BC to shrimp group [65,68]. There have been reports of *Pseudoalteromonas* genus being isolated with other genera including *Vibrio* [64,65]. Most species of this genus are used as probiotics in marine organisms such as shrimps [69–71] because they are noted for the production of diverse chemical compounds, including protease [72], amylases [73], β-galactosidases [74], phospholipases [75], and antimicrobial compounds [76]. Holmström [76] reported these bacteria to produce extracellular materials which inhibit the settlement and metamorphosis of ubiquitous fouling organisms including *Shewanella* which was observed to decrease with increasing inclusion of BC. This study revealed a significant increase in the abundance of *Pseudoalteromonas* in shrimps fed the BC3 diet. However, investigations of probiotic's effect on the microflora of *L. vannamei* are relatively rare. We therefore suggest that the reason for this phenomenon may be attributed to the probiotic's ability to produce antimicrobial agents to compete and outweigh the growth of other pathogenic bacteria, thus, increasing the abundance of beneficial bacteria in the gut as noted in other reports [59,60,77].

Digestive enzymes are known to break-down food and absorb

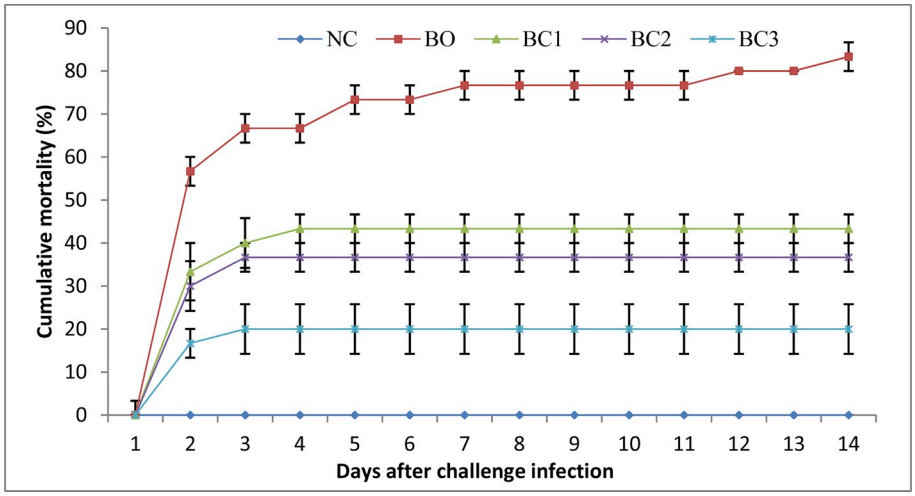

Fig. 9. Effects of dietary administration of BC on the post-challenge cumulative mortality percentage (A) of *L. vannamei* fry after infection with *V. parahaemolyticus*. NC = Negative control injected with PBS.

nutrients [78]. The digestive enzymes including Amylase (known to catalyze the hydrolysis of starch into sugars), lipase (known to catalyze the hydrolysis of fats (lipids)) and trypsin (catalyzes the hydrolysis of proteins into smaller peptides) [79,80] in this study significantly increased in the treated group compared to the untreated. Similar results of improved digestive enzyme activities in *Litopenaeus vannamei* [81] and *Fenneropenaeus indicus* [53] have been established. Verschuere et al. [14] in their work also noted that, *Bacillus* genus secrets a wide range of exoenzymes which aid in the nutritional enhancement of the host. Thus, the increase in digestive enzyme activities can be linked to the elevated beneficial bacteria such as *Pseudoalteromonas* in the treated groups since they secrete chemical compounds including protease, amylases, β-galactosidases, and phospholipases [72–75]. Moreover, the significant increase in the activities of trypsin, amylase and lipase in this current study for the treated groups compared to the control could buttress the reason why there were significant increase in the growth, feed utilization, whole body composition and immune response since digestive enzymes are noted for the enhancement of digestion and nutrient absorption in the gut.

Few research have reported, the physiological functions of the intestines to be developed alongside the morphological changes in the mucosal structure; thus, the higher or wider the intestinal epithelial cell microvilli, the vast absorptive surface area for higher amounts of nutrients uptake [68,82]. The result of the present study showed a significant increase in the VH and VW of the probiotic treated groups compared to the untreated which is supported by Ranadheera et al. [83].

Serum and liver immune substances (i.e., TP, ALB, GLO, ACP, TG, LYZ, SOD, GSH, GSH-Px, MDA, and TG) which are key players in defense mechanisms against infectious agents were analyzed. The TP and ALB except for GLO which had no significant difference though had higher elevations in the treated groups; were significantly enhanced in the treated groups compared to the untreated. This is in support to other findings [3,84,85]. ACP is also known to play a significant role in the immune system as a key compound of lysosomal enzymes to digest the invading organisms in invertebrate animals [86,87]. In the present study, the ACP activity showed significantly higher values in the BC2 and BC3 treated group compared to BC1 and BO. This result was not in accordance with earlier reports where dietary *B. subtilis* T13 could not significantly influence the immunity of sea cucumber in terms of ACP activity [87]. However, this result was consistent with Li et al. [88], who demonstrated significant enhancement of ACP activity in *Litopenaeus vannamei* using *Bacillus megeterium*. The exact mechanism of probiotics effects on aquatic animals concerning ACP activity is not so clear since less work has been done. The reason for this discrepancy might be due to the different bacterial species, different additive or dosage level and the different host specie. TG which is the main constituent of body fat in humans and other animals including shrimps in this experiment experienced a decreasing trend at an increasing probiotic supplementation with the least significant being observed in the BC3 group (Table 4). Li et al. [89] also observed a decreasing trend of TG with an increasing dose of probiotic though no significant difference was observed between treated and untreated groups. The reason for this inconsistency might be due to different culture conditions and additive used. Limited researches have been conducted on triglyceride in shrimps with respect to probiotic hence more need to be done.

LYZ is part of the innate immune system which functions by attacking, hydrolyzing and breaking glycosidic bonds in peptidoglycans which is the major component of gram-positive bacteria cell wall [90]. LYZ in the serum significantly enhanced BC treated groups compared to the untreated (Table 4), hence; we suggest that a dose of 1×10^8 CFU *B. coagulans* g^{-1} feed (BC3 group) should be used because it might be more effective. Zokaeifar et al. [81] reported similar results.

Antioxidant enzymes including SOD, GSH, and GSH-Px, help to shield host organisms' oxidative stress [91,92]. SOD is an enzyme that catalyzes the partitioning of reactive O_2^- to H_2O_2 [90]. GSH also is a low molecular mundificant capable of preventing damage to cellular components caused by reactive oxygen species including free radicals, heavy metals, peroxides and lipid peroxides [93] for which is then reduced back to GSH-Px using NADPH as electron donor once it's organized, whereas MDA reveals the toxic processes caused by free radicals [94]. The present study showed a significant enhancement in the SOD (serum) and GSH-Px (serum and liver) activities in the treated groups compared to the untreated. GSH had no significant difference in both serum and liver samples in all groups, yet higher elevation was recorded in the treated group than the untreated (Table 4). Similarly, enhanced activities of antioxidant enzymes using probiotics have been demonstrated [52,95,96].

Vibrio species have been reported to cause severe diseases in both humans and aquatic organisms [97–99]. Among the diseases that infect shrimps, the newly and severe acute hepatopancreatic necrosis disease (AHPND) which is caused by *V. parahaemolyticus* [100] since its first occurrence in China in 2009 [101], has caused lots of havoc such as reduction in production to the shrimp industry specifically in the affected Southeast Asian countries [102]. Using *Vibrio parahaemolyticus* in a fourteen-day challenge study after feeding trial resulted in a significant decrease in cumulative mortality of the treated group compared to the untreated. This observation in *L. vannamei* was in

agreement with previous investigations in *Litopenaeus vannamei* [50,81] and *Penaeus monodon* [66] and can thus be attributed to the high relative abundances of beneficial genus bacteria *Pseudoalteromonas* and enhanced immune response in the treated group.

This present study showed that the supplementation of potential probiotic *B. coagulans* to diet, significantly promote the growth performance, whole body composition, immune response and disease resistance in *L. vannamei*. Based on the findings shown in the different concentration of BC supplementation, we conclude that higher inclusion of BC supplementation at 1×10^8 CFU g^{-1} feed may provide health benefits by improving intestinal development and gut microflora, immune response and digestive enzyme activity.

Competing interests

The authors affirm that the experiment was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

We are grateful to the support given by Dr. E. D. Abarike. Also, the authors thank the laboratory members for experimental material preparation and all authors to whom we made reference to. This study was supported financially by the China Agriculture Research System (CARS-47), the Science and Technology Department, Guangdong Province (2015A020209170), Marine & Fisheries Department of Guangdong Province (A201608C06) and the Science and Technology Bureau of Zhanjiang (2016A3022).

References

[1] FAO, WHO, WHO Working Group Guidelines for the Evaluation of Probiotics in Food, (2002), https://doi.org/10.1111/j.1469-0691.2012.03873.
[2] K.L. Erickson, N.E. Hubbard, Probiotic immunomodulation in health and disease, J. Nutr. 130 (2000) 403S–409S.
[3] X. xia Zhou, Y. bo Wang, W. fen Li, Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities, Aquaculture 287 (2009) 349–353, https://doi.org/10.1016/j.aquaculture.2008.10.046.
[4] A. Irianto, B. Austin, Probiotics in aquaculture, J. Fish. Dis. 25 (2002) 633–642, https://doi.org/10.1046/j.1365-2761.2002.00422.x.
[5] L. Villamil, C. Tafalla, A. Figueras, B. Novoa, Evaluation of immunomodulatory effects of lactic acid bacteria in turbot (*Scophthalmus maximus*), Clin. Vaccine Immunol. 9 (2002) 1318–1323, https://doi.org/10.1128/CDLI.9.6.1318-1323.2002.
[6] S. Rengpipat, A. Tunyanun, A.W. Fast, S. Piyatiratitivorakul, P. Menasveta, Enhanced growth and resistance to Vibrio challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a Bacillus probiotic, Dis. Aquat. Org. 55 (2003) 169–173, https://doi.org/10.3354/dao055169.
[7] Y.B. Wang, Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*, Aquaculture 269 (2007) 259–264, https://doi.org/10.1016/j.aquaculture.2007.05.035.
[8] A. Rollo, R. Sulpizio, M. Nardi, S. Silvi, C. Orpianesi, M. Caggiano, A. Cresci, O. Carnevali, Live microbial feed supplement in aquaculture for improvement of stress tolerance, Fish Physiol. Biochem. 32 (2006) 167–177, https://doi.org/10.1002/jcp.26179.
[9] R. Fuller, History and development of probiotics, Probiotics (1992) 1–8, https://doi.org/10.1007/978-94-011-2364-8_1.
[10] H.A. Hong, H.D. Le, S.M. Cutting, The use of bacterial spore formers as probiotics, FEMS Microbiol. Rev. 29 (2005) 813–835, https://doi.org/10.1016/j.femsre.2004.12.001.
[11] A.D. Talpur, A.J. Memon, M.I. Khan, M. Ikhwanuddin, M.M. Daniel, Danish, A.B. Abol-Munafi, Inhibition of pathogens by lactic acid bacteria and application as water additive multi isolates in early stages larviculture of *P. pelagicus* (linnaeus, 1758), J. Anim. Plant Sci. 22 (2012) 54–64.
[12] G. Burr, D. Gatlin, S. Ricke, Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in Finnish aquaculture, J. World Aquacult. Soc. 36 (2005) 425–435, https://doi.org/10.1111/j.1749-7345.2005.tb00390.x.
[13] C.C. Lazado, C.M.A. Caipang, E.G. Estante, Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions, Fish Shellfish Immunol. 45 (2015) 2–12, https://doi.org/10.1016/j.fsi.2015.02.023.
[14] L. Verschuere, G. Rombaut, P. Sorgeloos, W. Verstraete, Probiotic bacteria as biological control agents in aquaculture, Microbiol. Mol. Biol. Rev. 64 (2000) 655–671, https://doi.org/10.1128/MMBR.64.4.655-671.2000.
[15] A. Panigrahi, V. Kiron, J. Puangkaew, T. Kobayashi, S. Satoh, H. Sugita, The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*, Aquaculture 243 (2005) 241–254, https://doi.org/10.1016/j.aquaculture.2004.09.032.
[16] A. Das, K. Nakhro, S. Chowdhury, D. Kamilya, Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*), Fish Shellfish Immunol. 35 (2013) 1547–1553, https://doi.org/10.1016/j.fsi.2013.08.022.
[17] C. Le Marrec, B. Hyronimus, P. Bressollier, B. Verneuil, M.C. Urdaci, Biochemical and genetic characterization of coagulin, a new antilisterial bacteriocin in the pediocin family of bacteriocins, produced by Bacillus coagulans I4, Appl. Environ. Microbiol. 66 (2000) 5213–5220, https://doi.org/10.1128/AEM.66.12.5213-5220.2000.
[18] A.E. Cazemier, S.F.M. Wagenaars, P.F. Ter Steeg, Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage bacilli, J. Appl. Microbiol. 90 (2001) 761–770, https://doi.org/10.1046/j.1365-2672.2001.01302.x.
[19] Y. Wang, L. Fu, J. Lin, Probiotic (*Bacillus coagulans*) cells in the diet benefit the white shrimp *Litopenaeus vannamei*, J. Shellfish Res. 31 (2012) 855–860, https://doi.org/10.2983/035.031.0333.
[20] Fao, World Fisheries and Aquaculture, (2012) doi:issn 10.
[21] C.H. Chiu, Y.K. Guu, C.H. Liu, T.M. Pan, W. Cheng, Immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*, Fish Shellfish Immunol. 23 (2007) 364–377, https://doi.org/10.1016/j.fsi.2006.11.010.
[22] FAO, The State of World Fisheries and Aquaculture 2012, FAO Fisheries technical paper, 2012 doi:92-5-105177-1.
[23] FAO, The State of World Fisheries and Aquaculture, (2014) doi:E-ISBN 978-92-5-108276-8.
[24] Z. Qi, X.H. Zhang, N. Boon, P. Bossier, Probiotics in aquaculture of China - current state, problems and prospect, Aquaculture 290 (2009) 15–21, https://doi.org/10.1016/j.aquaculture.2009.02.012.
[25] E. Bachère, Y. Gueguen, M. Gonzalez, J. De Lorgeril, J. Garnier, B. Romestand, Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*, Immunol. Rev. 198 (2004) 149–168, https://doi.org/10.1111/j.0105-2896.2004.00115.x.
[26] E.P. Nyangale, S. Farmer, D. Keller, D. Chernoff, G.R. Gibson, Effect of prebiotics on the fecal microbiota of elderly volunteers after dietary supplementation of *Bacillus coagulans* GBI-30, 6086, Anaerobe 30 (2014) 75–81 https://doi.org/10.1016/j.anaerobe.2014.09.002.
[27] M. Majeed, K. Nagabhushanam, S. Natarajan, A. Sivakumar, F. Ali, A. Pande, S. Majeed, S.K. Karri, *Bacillus coagulans* MTCC 5856 supplementation in the management of diarrhea predominant Irritable Bowel Syndrome: a double blind randomized placebo controlled pilot clinical study, Nutr. J. 15 (2016) 1–10, https://doi.org/10.1186/s12937-016-0140-6.
[28] J.C. Clemente, L.K. Ursell, L.W. Parfrey, R. Knight, The impact of the gut microbiota on human health: an integrative view, Cell 148 (2012) 1258–1270, https://doi.org/10.1016/j.cell.2012.01.035.
[29] D. Berry, C. Schwab, G. Milinovich, J. Reichert, K. Ben Mahfoudh, T. Decker, M. Engel, B. Hai, E. Hainzl, S. Heider, L. Kenner, M. Müller, I. Rauch, B. Strobl, M. Wagner, C. Schleper, T. Urich, A. Loy, Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis, ISME J. 6 (2012) 2091–2106, https://doi.org/10.1038/ismej.2012.39.
[30] AOAC, sixteenth ed., Official Methods of Analysis of AOAC International vol. 2, (1995).
[31] AOAC, Official Methods of Analysis 0f AOAC International, seventeenth ed., (2002), pp. 152–169, https://doi.org/10.1186/1756-3305-7-21 (Virginia, U.S.A., Arlington).
[32] A. Gupta, P. Gupta, A. Dhawan, Dietary supplementation of probiotics affects growth, immune response and disease resistance of *Cyprinus carpio* fry, Fish Shellfish Immunol. 41 (2014) 113–119, https://doi.org/10.1016/j.fsi.2014.08.023.
[33] A. Gupta, G. Verma, P. Gupta, Growth performance, feed utilization, digestive enzyme activity, innate immunity and protection against *Vibrio harveyi* of freshwater prawn, *Macrobrachium rosenbergii* fed diets supplemented with *Bacillus coagulans*, Aquacult. Int. (2016), https://doi.org/10.1007/s10499-016-9996-x.
[34] S. Tekinay, A.A. Davies, Dietary carbohydrate level influencing feed intake, nutrient utilisation and plasma glucose concentration in the rainbow trout, *Oncorhynchus mykiss*, Turk. J. Vet. Anim. Sci. 25 (2001) 657–666.
[35] Y. Suo, E. Li, T. Li, Y. Jia, J.G. Qin, Z. Gu, L. Chen, Response of gut health and microbiota to sulfide exposure in Pacific white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 63 (2017) 87–96, https://doi.org/10.1016/j.fsi.2017.02.008.
[36] T. Magoč, S.L. Salzberg, FLASH: fast length adjustment of short reads to improve genome assemblies, Bioinformatics 27 (2011) 2957–2963, https://doi.org/10.1093/bioinformatics/btr507.
[37] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Peña, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (2010) 335 https://doi.org/10.1038/nmeth.f.303.
[38] R.C. Edgar, UPARSE: highly accurate OTU sequences from microbial amplicon reads, Nat. Methods 10 (2013) 996–998, https://doi.org/10.1038/nmeth.2604.
[39] C.E. Shannon, The mathematical theory of communication, Bell Syst. Tech. J. 27

(1948) 379–423, https://doi.org/10.2307/3611062.
[40] W.F. Mahaffee, J.W. Kloepper, Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus L.*), Microb. Ecol. 34 (1997) 210–223, https://doi.org/10.1007/s002489900050.
[41] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, The folin by oliver, J. Biol. Chem. 193 (1951) 265–275, https://doi.org/10.1016/0304-3894(92)87011-4.
[42] D. Webster, A study of the interaction of bromocresol green with isolated serum globulin fractions, Clin. Chim. Acta 53 (1974) 109–115, https://doi.org/10.1016/0009-8981(74)90358-1.
[43] A.E. Ellis, Lysozyme assays, Tech. FIsh Immunol. 1 (1990) 101–103 https://xs.glgoo.top/scholar?hl=zh-CN&as_sdt=0%2C5&q=Ellis%2C+A.+E.+%281990%29+Lysozyme+Assays.+Techniques+in+Fish+Immunology%2C+101-103.+&btnG=.
[44] C.N. Bullerwell, S.A. Collins, S.P. Lall, D.M. Anderson, Growth performance, proximate and histological analysis of rainbow trout fed diets containing *Camelina sativa* seeds, meal (high-oil and solvent-extracted) and oil, Aquaculture 452 (2016) 342–350, https://doi.org/10.1016/j.aquaculture.2015.11.008.
[45] L.J. Reed, H. Muench, A simple method of estimating fifty per cent endpoints, Am. J. Epidemiol. 27 (1938) 493–497, https://doi.org/10.1093/oxfordjournals.aje.a118408.
[46] H. Liu, S. Wang, Y. Cai, X. Guo, Z. Cao, Y. Zhang, S. Liu, W. Yuan, W. Zhu, Y. Zheng, Z. Xie, W. Guo, Y. Zhou, Dietary administration of *Bacillus subtilis* HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, *Oreochromis niloticus*, Fish Shellfish Immunol. 60 (2017) 326–333, https://doi.org/10.1016/j.fsi.2016.12.003.
[47] A. Farzanfar, The use of probiotics in shrimp aquaculture, FEMS Immunol. Med. Microbiol. 48 (2006) 149–158, https://doi.org/10.1111/j.1574-695X.2006.00116.x.
[48] C.T. Buruiană, A.G. Profir, C. Vizireanu, Effects of probiotic bacillus species in aquaculture – an overview, Ann. Univ. Dunarea Jos Galati, Fascicle VI Food Technol. 38 (2014) 9–17.
[49] K. Vijayavel, M.P. Balasubramanian, Fluctuations of biochemical constituents and marker enzymes as a consequence of naphthalene toxicity in the edible estuarine crab *Scylla serrata*, Ecotoxicol. Environ. Saf. 63 (2006) 141–147, https://doi.org/10.1016/j.ecoenv.2005.02.004.
[50] J.L. Balcázar, T. Rojas-Luna, D.P. Cunningham, Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*, J. Invertebr. Pathol. 96 (2007) 147–150, https://doi.org/10.1016/j.jip.2007.04.008.
[51] I.E. Luis-Villaseñor, M.E. Macías-Rodríguez, B. Gómez-Gil, F. Ascencio-Valle, Á.I. Campa-Córdova, Beneficial effects of four Bacillus strains on the larval cultivation of *Litopenaeus vannamei*, Aquaculture 321 (2011) 136–144 https://doi.org/10.1016/j.aquaculture.2011.08.036.
[52] W.Y. Shen, L.L. Fu, W.F. Li, Y.R. Zhu, Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*), Aquacult. Res. 41 (2010) 1691–1698, https://doi.org/10.1111/j.1365-2109.2010.02554.x.
[53] S. Ziaei-Nejad, M.H. Rezaei, G.A. Takami, D.L. Lovett, A.-R. Mirvaghefi, M. Shakouri, The effect of *Bacillus spp*. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*, Aquaculture 252 (2006) 516–524 https://doi.org/10.1016/j.aquaculture.2005.07.021.
[54] S. Saad, M. Habashy, M. Sharshar, Growth response of the freshwater prawn, *Macrobrachium rosenbergii* (De Man), to diets having different levels of Biogen®, World Appl. Sci. J. 6 (2009) 550–556.
[55] T. Boonthai, V. Vuthiphandchai, S. Nimrat, Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaeus monodon*), Aquacult. Nutr. 17 (2011) 634–644, https://doi.org/10.1111/j.1365-2095.2011.00865.x.
[56] S.P. Claus, H. Guillou, S. Ellero-Simatos, The gut microbiota: a major player in the toxicity of environmental pollutants? Npj Biofilms Microbiomes (2016) 16003, https://doi.org/10.1038/npjbiofilms.2016.3.
[57] N. Kar, K. Ghosh, Enzyme producing bacteria in the gastrointestinal tracts of *Labeo rohita* (Hamilton) and *Channa punctatus* (Bloch), Turkish, J. Fish. Aquat. Sci. (2008) 115–120.
[58] R.F. Ramirez, B.A. Dixon, Enzyme production by obligate intestinal anaerobic bacteria isolated from oscars (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*) and southern flounder (*Paralichthys lethostigma*), Aquaculture 227 (2003) 417–426, https://doi.org/10.1016/S0044-8486(03)00520-9.
[59] J.K. Spinler, M. Taweechotipatr, C.L. Rognerud, C.N. Ou, S. Tumwasorn, J. Versalovic, Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens, Anaerobe 14 (2008) 166–171, https://doi.org/10.1016/j.anaerobe.2008.02.001.
[60] E.F. O'shea, P.D. Cotter, C. Stanton, R.P. Ross, C. Hill, Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid, Int. J. Food Microbiol. 152 (2012) 189–205, https://doi.org/10.1016/j.ijfoodmicro.2011.05.025.
[61] S. Miao, C. Zhao, J. Zhu, J. Hu, X. Dong, L. Sun, Dietary soybean meal affects intestinal homoeostasis by altering the microbiota, morphology and inflammatory cytokine gene expression in northern snakehead, Sci. Rep. 8 (2018) 113, https://doi.org/10.1038/s41598-017-18430-7.
[62] M.C. Collado, J. Meriluoto, S. Salminen, Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus, Lett. Appl. Microbiol. 45 (2007) 454–460, https://doi.org/10.1111/j.1472-765X.2007.02212.x.
[63] F. Gao, F. Li, J. Tan, J. Yan, H. Sun, Bacterial community composition in the gut content and ambient sediment of sea cucumber *Apostichopus japonicus* revealed by 16S rRNA gene pyrosequencing, PLoS One 9 (2014) 1–10, https://doi.org/10.1371/journal.pone.0100092.
[64] J.T. Tzuc, D.R. Escalante, R. Rojas Herrera, G. Gaxiola Cortés, M.L.A. Ortiz, Microbiota from Litopenaeus vannamei: digestive tract microbial community of Pacific white shrimp (*Litopenaeus vannamei*), SpringerPlus 3 (2014) 1–10, https://doi.org/10.1186/2193-1801-3-280.
[65] A.P.A. Oxley, W. Shipton, L. Owens, D. McKay, Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*, J. Appl. Microbiol. 93 (2002) 214–223, https://doi.org/10.1046/j.1365-2672.2002.01673.x.
[66] K. Ramalingam, D.R. Shyamala, Influence of *Vibrio parahaemolyticus* MTCC 451 on the levels of ascorbic acid and histamine in *Penaeus monodon* (Fabricius), J. Environ. Biol. 27 (2006) 67–70.
[67] M.N. Kim, H.J. Bang, Detection of marine pathogenic bacterial Vibrio species by multiplex polymerase chain reaction (PCR), J. Environ. Biol. 29 (2008) 543–546.
[68] J. Romero, E. Ringø, D.L. Merrifield, The Gut Microbiota of Fish, Aquacult. Nutr. (2014) 75–100, https://doi.org/10.1002/9781118897263.ch4.
[69] G. Le Moullac, E. Goyard, D. Saulnier, P. Haffher, E. Thouard, G. Nedelec, J. Goguenheim, C. Rouxel, G. Cuzon, Recent improvements in broodstock management and larviculture in marine species in Polynesia and New Caledonia: genetic and health approaches, Aquaculture 227 (2003) 89–106, https://doi.org/10.1016/S0044-8486(03)00497-6.
[70] A.D. Schulze, A.O. Alabi, A.R. Tattersall-Sheldrake, K.M. Miller, Bacterial diversity in a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains, Aquaculture 256 (2006) 50–73, https://doi.org/10.1016/j.aquaculture.2006.02.008.
[71] K.I. ten Doeschate, V.E. Coyne, Improved growth rate in farmed *Haliotis midae* through probiotic treatment, Aquaculture 284 (2008) 174–179, https://doi.org/10.1016/j.aquaculture.2008.07.018.
[72] K. Venkateswaran, N. Dohmoto, *Pseudoalteromonas peptidolytica sp*. nov., a novel marine mussel-thread- degrading bacterium isolated from the Sea of Japan, Int. J. Syst. Evol. Microbiol. 50 (2000) 565–574, https://doi.org/10.1099/00207713-50-2-565.
[73] L. Gavrilovic, R.W. O'Brien, R.L. Sanders, Secretion of amylase by the marine bacterium, *Alteromonas rubra*, Aust. J. Biol. Sci. 35 (1982) 111–124, https://doi.org/10.1071/BI9820111.
[74] A. Hoyoux, I. Jennes, P. Dubois, S. Genicot, F. Dubail, J.M. François, E. Baise, G. Feller, C. Gerday, Cold-adapted β-galactosidase from the *antarctic psychrophile Pseudoalteromonas haloplanktis*, Appl. Environ. Microbiol. 67 (2001) 1529–1535, https://doi.org/10.1128/AEM.67.4.1529-1535.2001.
[75] E. Cadman, J. Eichberg, The presence of phospholipase a and lysophospholipase activities in culture supernatant fluid from *Alteromonas espejiana*, Int. J. Biochem. 15 (1983) 1155–1159, https://doi.org/10.1016/0020-711X(83)90231-8.
[76] C. Holmström, S. Egan, A. Franks, S. McCloy, S. Kjelleberg, Antifouling activities expressed by marine surface associated *Pseudoalteromonas species*, FEMS Microbiol. Ecol. 41 (2002) 47–58, https://doi.org/10.1016/S0168-6496(02)00239-8.
[77] H.E. Samli, S. Dezcan, F. Koc, M.L. Ozduven, A.A. Okur, N. Senkoylu, Effects of *Enterococcus faecium* supplementation and floor type on performance, morphology of erythrocytes and intestinal microbiota in broiler chickens, Br. Poult. Sci. 51 (2010) 564–568, https://doi.org/10.1080/00071668.2010.507241.
[78] N. Gobi, B. Vaseeharan, J.-C. Chen, R. Rekha, S. Vijayakumar, M. Anjugam, A. Iswarya, Dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 improves growth performance, mucus and serum immune parameters, antioxidant enzyme activity as well as resistance against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*, Fish Shellfish Immunol. 74 (2018) 501–508 https://doi.org/10.1016/j.fsi.2017.12.066.
[79] A. Rawlings, Neil, Barrett, Families of serine peptidases, Methods Enzymol. 244 (1994) 19–61, https://doi.org/10.1016/0076-6879(94)44004-2.
[80] A. Svendsen, Lipase protein engineering, Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1543 (2000) 223–238, https://doi.org/10.1016/S0167-4838(00)00239-9.
[81] H. Zokaeifar, J.L. Balcázar, C.R. Saad, M.S. Kamarudin, K. Sijam, A. Arshad, N. Nejat, Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*, Fish Shellfish Immunol. 33 (2012) 683–689, https://doi.org/10.1016/j.fsi.2012.05.027.
[82] M. Kristiansen, D.L. Merrifield, J.L.G. Vecino, R. Myklebust, E. Ringø, Evaluation of prebiotic and probiotic effects on the intestinal gut microbiota and histology of atlantic salmon (*Salmo salar L.*), J. Aquacult. Res. Dev. s1 (2011) 1–8, https://doi.org/10.4172/2155-9546.S1-009.
[83] C.S. Ranadheera, C.A. Evans, M.C. Adams, S.K. Baines, Effect of dairy probiotic combinations on in vitro gastrointestinal tolerance, intestinal epithelial cell adhesion and cytokine secretion, J. Funct. Foods. 8 (2014) 18–25, https://doi.org/10.1016/j.jff.2014.02.022.
[84] Y.B. Wang, Z.Q. Tian, J.T. Yao, W. fen Li, Effect of probiotics, *Enteroccus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response, Aquaculture 277 (2008) 203–207, https://doi.org/10.1016/j.aquaculture.2008.03.007.
[85] S. Mg, K. Ammani, Effect of probiotic bacterium on growth and biochemical parameters of shrimp *Litopenaeus vannamei*, Int. J. Recent Sci. Res. 6 (2015) 2871–2875.
[86] C. Yang, J. Kong, Q. Wang, Q. Liu, Y. Tian, K. Luo, Heterosis of haemolymph analytes of two geographic populations in Chinese shrimp *Fenneropenaeus chinensis*, Fish Shellfish Immunol. 23 (2007) 62–70, https://doi.org/10.1016/j.fsi.2006.09.005.
[87] Y.C. Zhao, W.B. Zhang, W. Xu, K.S. Mai, Y.J. Zhang, Z.G. Liufu, Effects of potential probiotic *Bacillus subtilis* T13 on growth, immunity and disease resistance against

Vibrio splendidus infection in juvenile sea cucumber *Aposticho pus japonicus*, Fish Shellfish Immunol. 32 (2012) 750–755, https://doi.org/10.1016/j.fsi.2012.01.027.

[88] J. Li, B. Tan, K. Mai, Dietary probiotic *Bacillus OJ* and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*), Aquaculture 291 (2009) 35–40, https://doi.org/10.1016/j.aquaculture.2009.03.005.

[89] J.P. Apún-Molina, A. Santamaría-Miranda, A. Luna-González, J.C. Ibarra-Gámez, V. Medina-Alcantar, I.S. Racotta, Growth and metabolic responses of whiteleg shrimp *Litopenaeus vannamei* and Nile tilapia *Oreochromis niloticus* in polyculture fed with potential probiotic microorganisms on different schedules, Lat. Am. J. Aquat. Res. 43 (2015) 435–445, https://doi.org/10.3856/vol43-issue3-fulltext-5.

[90] J. Li, Y. Xu, L. Jin, X. Li, Effects of a probiotic mixture (*Bacillus subtilis* YB-1 and *Bacillus cereus* YB-2) on disease resistance and non-specific immunity of sea cucumber, *Apostichopus japonicus* (Selenka), Aquacult. Res. 46 (2015) 3008–3019, https://doi.org/10.1111/are.12453.

[91] R. Kohen, A. Nyska, Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification, Toxicol. Pathol. 30 (2002) 620–650, https://doi.org/10.1080/01926230290166724.

[92] R.M. Martínez-Álvarez, A.E. Morales, A. Sanz, Antioxidant defenses in fish: biotic and abiotic factors, Rev. Fish Biol. Fish. 15 (2005) 75–88, https://doi.org/10.1007/s11160-005-7846-4.

[93] A. Pompella, A. Visvikis, A. Paolicchi, V. De Tata, A.F. Casini, The changing faces of glutathione, a cellular protagonist, Biochem. Pharmacol. 66 (2003) 1499–1503, https://doi.org/10.1016/S0006-2952(03)00504-5.

[94] N. Couto, N. Malys, S.J. Gaskell, J. Barber, Partition and turnover of glutathione reductase from *Saccharomyces cerevisiae*: a proteomic approach, J. Proteome Res. 12 (2013) 2885–2894, https://doi.org/10.1021/pr4001948.

[95] L. Weifen, Z. Xiaoping, S. Wenhui, D. Bin, L. Quan, F. Luoqin, Z. Jiajia, W. yue, Y. Dongyou, Effects of Bacillus preparations on immunity and antioxidant activities in grass carp (*Ctenopharyngodon idellus*), Fish Physiol. Biochem. 38 (2012) 1585–1592, https://doi.org/10.1007/s10695-012-9652-y.

[96] E.D. Abarike, J. Cai, Y. Lu, H. Yu, L. Chen, J. Jian, J. Tang, L. Jun, F.K.A. Kuebutornye, Effects of a commercial probiotic BS containing *Bacillus subtilis* and *Bacillus licheniformis* on growth, immune response and disease resistance in Nile tilapia, *Oreochromis niloticus*, Fish Shellfish Immunol. 82 (2018) 229–238, https://doi.org/10.1016/j.fsi.2018.08.037.

[97] Y. Yoon, K. Im, Y. Koh, S. Kim, J. Kim, Genotyping of six pathogenic Vibrio species based on RFLP of 16S rDNAs for rapid identification, Society 41 (2003) 312–319.

[98] H.H. Sung, S.F. Hsu, C.K. Chen, Y.Y. Ting, W.L. Chao, Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of Vibrio communities in pond water and shrimp hepatopancreas during cultivation, Aquaculture 192 (2001) 101–110, https://doi.org/10.1016/S0044-8486(00)00458-0.

[99] S.-W. Hsu, J.-C. Chen, The immune response of white shrimp *Penaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under sulfide stress, Aquaculture 271 (2007) 61–69, https://doi.org/10.1016/j.aquaculture.2007.05.028.

[100] L. Tran, L. Nunan, R.M. Redman, L.L. Mohney, C.R. Pantoja, K. Fitzsimmons, D.V. Lightner, Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp, Dis. Aquat. Org. 105 (2013) 45–55, https://doi.org/10.3354/dao02621.

[101] NACA-FAO, Quarterly Aquatic Animal Disease Report (Asia and Pacific Region) 2011/2, July – September, (2012).

[102] FAO/MARD, Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (Under TCP/VIE/3304), (2015) http://www.fao.org/docrep/018/i3422e/i3422e.pdf.