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Effects of supplementation with freeze-dried *Clostridium butyricum* powder after replacement of fishmeal with cottonseed protein concentrate on growth performance, immune response, and intestinal microbiota of *Litopenaeus vannamei*

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Abstract

The present study was designed to investigate the effects of supplementation with freeze-dried *Clostridium butyricum* (CB) powder on the growth, immune function and intestinal health of *Litopenaeus vannamei* after replacing fishmeal in the diet with cottonseed protein concentrate (CPC). Six treatment groups were designed, namely the control group (CON, 25% fish meal) and five alternative groups (CPC replacing 40% fishmeal protein in the control group). Based on the alternative group, 0%, 0.065%, 0.26%, 1.04%, and 4.16% of freeze-dried CB bacterial powder (4.6×10^8 CFU/g) were added, recorded as CB 0, CB 0.065, CB 0.26, CB 1.04, and CB 4.16, respectively. Each treatment had 3 replicates of 40 shrimps (0.29 ± 0.01 g) each and breeding for 8 weeks. After the experiment, serum enzyme activities, muscle amino acids, and intestinal parameters (short-chain fatty acids, digestive enzymes, gene expression, and microbiota) were tested to explore the effects of freeze-dried CB powder in shrimp aquaculture. The results showed that the CB1.04 group had the highest final body weight, weight gain rate, and specific growth ratio ($P > 0.05$). Freeze-dried CB powder increased the activity of serum superoxide dismutase, glutathione peroxidase, complement 3, and complement 4. Muscle tyrosine, proline, and total essential amino acids were remarkably increased in the CB 1.04 group ($P < 0.05$). Propionic acid levels were elevated in the CB 1.04 and CB4.16 groups ($P < 0.001$). The relative expression of Dorsal, Relish, and Target of Rapamycin (*TOR*) genes was significantly increased in the CB 1.04 group ($P < 0.01$). Actinobacteria and *Demequina* abundance was significantly higher in the CB 1.04 group ($P < 0.01$). The results of the *Vibrio parahaemolyticus* challenge test showed the highest cumulative mortality rate (43.33%) in the CB0 group and the lowest cumulative mortality rate (20%) in the CB1.04 group. This study confirmed that freeze-dried CB powder alleviated the negative effects of CPC replacement of fish meal protein in *Litopenaeus vannamei*, and the optimum additive level was 2.11% (9.71×10^9 CFU/kg) as indicated by binary regression analysis of specific growth ratio.

Keywords *Litopenaeus vannamei*, *Clostridium butyricum*, Immunity, Intestinal microbiota

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Introduction

With the growth of intensive aquaculture, global antibiotics consumption has been on the rise to control disease outbreak [66]. However, the indiscriminate of antibiotics results in antibiotic residues in aquatic products and bacterial resistance, causing economic losses and posing threats to the environment and human health [81]. Moreover, the ever-growing gap between fishmeal supply and human demand for aquatic products is becoming increasingly prominent. Since the contribution of fishmeal to growth performance in cultured fish lies in its high protein level, intensive research is being conducted to identify alternative protein sources for fishmeal [44]. Plant proteins have been promoted as ideal protein sources to replace fishmeal by virtue of their positive characteristics of competitive price, wide availability, and stable yields [56]. Cottonseed protein concentrate (CPC) is processed from cottonseed, a by-product of cotton. In recent years CPC has been considered a high-quality plant protein source for sustainable aquaculture due to its high annual yield and better nutritional content than soybeans [79]. CPC (gossypol content is 0.0079 g/kg, tested by SGS, China) undergoes processing methods such as solvent extraction, heat treatment, and fermentation to reduce anti-nutritional factors like gossypol [56]. These treatments significantly decrease the levels of free gossypol, making CPC a more viable and safer alternative to fishmeal in aquaculture diets. In *Larimichthys crocea*, CPC replacing less than 50% of fishmeal did not affect the intestinal digestive enzyme activity, while excessive inclusion of CPC (exceeding 50%) impaired intestinal digestion and absorption, resulting in a decrease in the average intestinal circumference, muscle thickness, and intestinal villus height [64]. High levels of CPC replacement reduced the activity of intestinal antioxidant enzymes such as superoxide dismutase and catalase, as well as total antioxidant enzymes in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) [79]. Replacing 15% and 30% of fish meal with CPC in the diets of largemouth bass (*Micropterus salmoides*) had no significant effect on WGR and FCR. However, when 45% and 60% of fish meal were replaced, the WFR decreased and the FCR increased [77]. Probiotic supplementation is now recognised as a promising strategy to reduce the risk of plant protein substitution [45].

Probiotics play a crucial role in sustainable aquaculture by significantly increasing feed utilization, improving digestion and absorption, as well as enhancing immunity in aquatic animals [6]. To preserve the viability of probiotics for a long period of time, it is necessary to keep them in a dry state. Currently, the conventional process for the production of dried probiotics is freeze-drying [4]. Freeze-drying technology not only facilitates the transportation, marketing, and application of probiotics, but also reduces external exposure of organisms during storage [54]. However, the bacterial viability may be reduced due to various factors during the freeze-drying process. Hence, freeze-drying protectants are added to reduce the damage to cells during freeze-drying [41]. The preferred freeze-drying protectants are sugars (lactose, glucose, β-cyclodextrin, etc.), inorganic salts (phosphate, calcium carbonate, sodium acetate, etc.), and complexes (skimmed milk, gelatin), which protect the bacteria from external damage in different ways [34, 63]. Gelatin, a mixture of polypeptide molecules with a broad molecular weight distribution, is the hydrolysis product of collagen proteins and excellent water-holding, film-forming and gelling properties [24]. Gelatin can not only be used as a wall material for the microencapsulation of probiotics, but also as a protective agent for the freeze-drying of probiotics [39].

Clostridium butyricum (CB)is a strictly anaerobic bacterium widely present in the gut and faeces of humans and animals. CB has the ability to break down carbohydrates, and the enzyme amylase can break down undigested starch into oligosaccharides, which makes starch more readily available for absorption by the host [65]. CB plays a crucial role in modulating the gut microbiota composition by promoting the growth of beneficial bacteria and inhibiting pathogenic microorganisms through the production of bacteriocins and organic acids. This modulation helps maintain gut microbial homeostasis and enhances overall gastrointestinal health [75]. The short-chain fatty acids (SCFAs) in CB metabolites can enhance immune cell growth and proliferation, maintain intestinal acid-base balance, and promote intestinal peristalsis [42]. The production of butyric acid by CB is another key aspect of its probiotic function. Butyric acid serves as a primary energy source for colonocytes, promotes intestinal epithelial cell proliferation, and induces apoptosis in colon cancer cells, indicating potential anticarcinogenic properties [76]. CB also exhibits significant anti-inflammatory properties. It modulates the host immune response by increasing the production of anti-inflammatory cytokines like interleukin-10 and decreasing pro-inflammatory cytokines such as tumor necrosis factor-alpha and interleukin-6 [52]. This immunomodulatory effect contributes to alleviating inflammatory bowel diseases and other inflammation-related disorders. The addition of CB at 10^7 CFU/g to *Scophthalmus maximus* diets significantly facilitated growth performance, improved intestinal morphology, and suppressed intestinal inflammation [3]. Similarly, the addition of 0.2% and 0.3% CB to perch diets significantly increased SGR and WG, enhanced intestinal amylase activity, and reduced the abundance of harmful intestinal bacteria [23].

Dietary supplementation with CB B3 at 3.0×10^7 CFU/g significantly enhanced growth performance, digestive enzyme activities, immune response, lipid metabolism, and modulated gut microbiota in juvenile yellow catfish (*Pelteobagrus fulvidraco*) [83]. The previous study demonstrated that dietary supplementation with CB cultures in high-carbohydrate diets significantly improved carbohydrate utilization, enhanced hepatic antioxidant capacity, upregulated beneficial metabolic and antioxidant gene expressions, and favorably modulated the intestinal microbiota in largemouth bass, thereby mitigating the adverse effects of high carbohydrate intake [62]. CB has been isolated from the intestine of *Litopenaeus vannamei* in a previous work by our group, and liquid CB has been utilized to conduct dietary spray breeding experiments [73]. Whereas, the preparation of freeze-dried powder of CB has not been reported. Therefore, this study aims to provide theoretical and technical basis for the application of freeze-dried CB as a probiotic supplement in aquaculture diets. The findings are expected to enhance the nutritional value of CPC-based diets, improve intestinal microbiota balance, enhance immunity, and promote sustainable and efficient development of *Litopenaeus vannamei* aquaculture.

Materials and methods

Preparation of experimental diets

Clostridium butyricum LV1 (the accession number: OQ216736) was previously isolated by our group from the intestine of *Litopenaeus vannamei*. Briefly, the freeze-dried CB powder (4.6×10^8 CFU/g) was prepared as follows: First, the CB bacterial precipitate was collected. Then, the sterilized compound protectant solution (0.5% dipotassium hydrogen phosphate, 8% glucose, 12% β-cyclodextrin, and 15% skimmed milk; lyophilised protectant ratios derived from orthogonal tests) was added to the bacterial precipitate in the proportion of 1/10 of the volume of the original fermentation broth. Finally, 2% gelatin wall material solution was added and mixed well. After completion of the above steps, it was stored at -80 °C for 2 h and then freeze-dried to a dry powder. The detailed process of CB powder production is described in Supplementary material 1. The experimental diets were formulated as shown in Table 1. Dietary amino acid

Table 1 Composition and nutrient content of the experimental diet (dry matter, %)

Ingredient	CON	CB 0	CB 0.065	CB 0.26	CB 1.04	CB 4.16
Fish meal	25.00	15.00	15.00	15.00	15.00	15.00
Concentrated cottonseed protein	0.00	11.27	11.27	11.27	11.27	11.27
Shrimp shell meal	6.00	6.00	6.00	6.00	6.00	6.00
Dehulled soybean meal	10.00	10.00	10.00	10.00	10.00	10.00
Corn gluten meal	10.00	10.00	10.00	10.00	10.00	10.00
Peanut meal	10.00	10.00	10.00	10.00	10.00	10.00
Wheat flour	25.00	25.00	25.00	25.00	25.00	25.00
Fish oil	1.50	2.02	2.02	2.02	2.02	2.02
Soybean lecithin	2.50	2.50	2.50	2.50	2.50	2.50
Premix[1)]	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
Vc	0.10	0.10	0.10	0.10	0.10	0.10
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00	2.00	2.00
Attractant	0.10	0.10	0.10	0.10	0.10	0.10
Antioxidant	0.05	0.05	0.05	0.05	0.05	0.05
MCC	6.25	4.22	4.16	3.96	3.18	0.06
Methionine	0.00	0.07	0.07	0.07	0.07	0.07
Lysine	0.00	0.17	0.17	0.17	0.17	0.17
Freeze-dried *Clostridium butyricum*	0.00	0.00	0.065	0.26	1.04	4.16
Total	100	100	100	100	100	100
Crude protein	39.31	39.68	39.59	40.11	40.04	39.89
Crude lipid	7.34	6.18	5.94	6.14	6.08	6.16
Moisture	9.10	9.80	10.00	9.40	9.20	10.00

1) Premix offered by Qingdao Master Biotech Co., Ltd

Vitamin and Mineral Premix (/Kg) includes following: Vitamin A, 500000IU; Vitamin D3, 100000IU; Vitamin E, 4000 mg; Vitamin K3, 1000 mg; Thiamine, 500 mg; Riboflavin, 1000 mg; Pyridoxine, 1000 mg; Cobalamin, 2 mg; Niacin, 4000 mg; Calcium pantothenate, 2000 mg; Folic acid, 100 mg; Vitamin C, 15,000 mg; Iron, 10,000 mg; Copper, 300 mg; Zinc, 5000 mg; Manganese, 1200 mg; Iodine, 80 mg; Selenium, 30 mg; Cobalt, 20 mg

composition is shown in Table 2. Six isonitrogenous and isolipid diets were prepared. The control group (CON) contained 25% fishmeal protein. The experimental group utilized CPC to substitute 40% of the fishmeal protein in the control group, designated as CB0. Based on the CB 0 group, 0.065%, 0.26%, 1.04%, and 4.16% of freeze-dried CB powder were added and recorded as CB 0.065 (3×10^8 CFU/kg), CB 0.26 (1.2×10^9 CFU/kg), CB 1.04 (4.8×10^9 CFU/kg), and CB 4.16 (1.92×10^{10} CFU/kg), respectively. According to the formula proportions, each component was precisely weighed and mixed in a V-mixer. Then add the pre-measured fish oil, soy lecithin and pure water and mix thoroughly. A twin-screw extruder (F-26, South China University of Technology) was employed to produce diets with a particle size of 1.5 mm. These diets were then placed in a 60 °C oven to dry and mature for 30 min before being utilized following natural air-drying in the room.

Laboratory animal and breeding management

Litopenaeus vannamei were sourced from ZhanJiang Hisenor Marine Biotechnology Co., Ltd. Shrimp breeding trials were conducted at Zhanjiang Marine Hi-Tech Park (Zhanjiang, China). A total of 720 healthy juvenile shrimps (0.29 ± 0.01 g) were selected and randomly divided into six treatments with three replicates of 40 juvenile shrimps per replicate. Feeding took place four times a day, at 7:00, 11:00, 17:00 and 21:00. Eating behavior was observed 40 min post-feeding, and feeding amounts were adjusted accordingly. Daily records of feeding quantities were maintained throughout the 8-week aquaculture cycle.

Sample collection and analysis

At the end of the breeding period, all shrimps were starved for 24 h prior to sampling. The total number of shrimp and their body weights in each tank (0.3 m^3) were recorded to calculate the final body weight (FBW), weight gain rate (WGR), special growth rate (SGR), survival rate (SR), and feed conversion rate (FCR). Growth indicator calculations were performed as described by Wang et al. [68].

Three shrimp were randomly sampled from each replicate for whole-body composition analysis. The conventional composition of the diet and shrimp was analysed using the AOAC standard method [67]. Crude protein, crude lipid and ash in the samples were determined by Kjeldahl nitrogen fixation, Soxhlet extraction and incineration at 550 °C in a muffle furnace, respectively. The samples were dried in a 105 °C drying oven until constant weight to measure moisture.

Ten shrimp were randomly taken from each replicate and haemolymph was extracted from the ventral sinus of the first abdominal segment using a 1 ml sterile syringe. Subsequently placed in a 1.5 mL centrifuge tube, serum was collected and stored at -80 °C for enzyme activity analysis. Serum total antioxidant capacity (T-AOC, ml541001), malondialdehyde (MDA, ml555268), superoxide dismutase (SOD, ml926247), glutathione peroxidase (GPX, ml113425), complement 4 (C4, ml003461),

Table 2 Amino acid composition of experimental diets

Amino acids (%)	Diets					
	CON	CB 0	CB 0.065	CB 0.26	CB 1.04	CB 4.16
Threonine	1.39	1.37	1.34	1.36	1.36	1.35
Valine	1.59	1.57	1.58	1.58	1.60	1.59
Methionine	0.80	0.78	0.80	0.78	0.79	0.80
Isoleucine	1.47	1.42	1.44	1.40	1.41	1.42
Leucine	3.20	3.16	3.12	3.15	3.14	3.15
Phenylalanine	1.73	1.74	1.76	1.76	1.77	1.76
Lysine	2.11	2.05	2.04	2.07	2.06	2.09
Histidine	1.03	1.00	1.01	1.01	1.02	1.01
Arginine	2.13	2.51	2.53	2.55	2.54	2.50
Aspartic acid	3.35	3.35	3.36	3.39	3.40	3.38
Serine	1.65	1.66	1.57	1.63	1.66	1.64
Glutamic acid	6.91	7.33	7.34	7.37	7.42	7.32
Glycine	1.86	1.80	1.78	1.81	1.82	1.80
Alanine	2.21	2.11	2.06	2.12	2.10	2.09
Tyrosine	1.27	1.30	1.27	1.30	1.24	1.25
Proline	2.21	2.22	2.25	2.26	2.27	2.24

and complement 3 (C3, ml003460) were determined according to the commercial kits instructions of Shanghai Enzyme-linked Biotechnology Co., Ltd (China). Acid phosphatase (ACP, A060-2-2) and alkaline phosphatase (AKP, A059-2-2) activity in serum were measured according to the instructions of the commercial kits (Nanjing Jiancheng Biological Engineering Institute, China).

Three shrimp intestines (midgut) were randomly sampled from each culture tank for digestive enzyme activity assay. Saline and tissue samples were weighed and ground in a 1:9 ratio, followed by centrifugation to collect the supernatant. Activities of Intestinal trypsin (TRY, ml036384), amylase (AMS, ml922299) and lipase (LIP, ml036371) were determined using commercial kits (Shanghai Enzyme-Link Biotechnology Co., Ltd.), following prescribed methods.

Muscles from five shrimp were randomly collected from each replicate and freeze-dried to determine amino acid content. The composition of muscle amino acids was determined following the GB 5009.124–2016 standard (National Standards of the People's Republic of China). Each sample was treated with hydrochloric acid solution at a concentration of 6 mol/L, frozen, filled with nitrogen, followed by hydrolysis at 110 °C for 22 h. Subsequently, the amino acid content was determined using a fully automatic amino acid analyser (model LA8080, Hitachi, Japan).

Furthermore, six shrimp intestines (midgut) were randomly obtained from each breeding tank for intestinal flora sequencing analysis. Eight shrimp intestines were randomly selected from each culture tank, immediately placed in liquid nitrogen, and then stored at -80 °C for determination of intestinal short-chain fatty acids (SCFAs). Three shrimp were randomly selected from each replicate, rapidly stripped of their intestines, and placed in RNA-later-free Eppendorf tubes containing RNA-later. The samples were then stored at -80 °C after complete infiltration at 4 °C for subsequent analysis of shrimp immunity and growth-related gene expression.

Analysis of the structure of intestinal flora

The sequencing of the intestinal flora was completed by Gene Denovo Biotechnology Co., Ltd (Guangzhou, China). Shrimp intestinal bacterial DNA was extracted according to the HiPure Soil DNA kit instructions. The concentration and quality of the resulting DNA were assessed using 0.8% agarose gel electrophoresis. Primers 341 F and 806R sequences were utilized to amplify the V3-V4 variable region of the bacterial 16 S rDNA gene. Sequencing was performed on a Novaseq 6000 with PE250 mode pooling. Subsequently, the obtained data underwent quality control, clustering, and de-chimerised using the Usearch method to generate operational taxonomic units (OTUs). Bioinformatic analyses, including species composition, indicator species, alpha diversity, beta diversity, and functional prediction, were conducted based on OTUs and species abundance tables.

Determination of intestinal short-chain fatty acids

Intestinal SCFAs measurement was accomplished by BioTech Pack Technology Company Ltd. Specific experimental steps are shown in Supplementary Material 1.

Extraction of intestinal RNA and analysis by real-time fluorescence quantitative PCR

Total intestinal RNA was extracted using the TransZol Up Plus RNA kit (TransGen Biotech, Beijing, China). Briefly, frozen samples were weighed and immediately ground into a fine powder under liquid nitrogen, then homogenized in TransZol Up and RNA Extraction Agent, followed by phase separation to collect the aqueous phase containing RNA. The RNA was purified using spin columns with sequential washes, eluted with RNase-free water, and stored at −80 °C for further analysis. The integrity of the extracted total RNA was evaluated using 1% agarose gel electrophoresis, and the purity and concentration were determined with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, USA). This ensured that the RNA samples used in subsequent experiments exhibited clear and intact bands during agarose gel electrophoresis, with OD260/OD280 ratios between 1.8 and 2.0. cDNA was synthesized with the Evo M-MLV reverse transcription premix kit (Accurate Biotechnology Ltd., Guangzhou, China). Genomic DNA was first removed from the 10 μl reaction system, incubated at 42 °C for 2 min. Subsequently, a reverse transcription reaction occurred in a 20 μl reaction system (10 μl: Step 1 reaction solution; 4 μl: 5×Evo M-MLV RT Reaction Mix; 6 μl: RNase-free water). The reaction conditions include incubation at 37℃ for 15 min, followed by 85 °C for 5 s. Roche LightCycler 480 II (Roche, Switzerland) was used for PCR reactions. The specific primer sequences used in this experiment are provided in Table 3. The $2^{-\Delta\Delta CT}$ method was employed to compare the relative expression levels of the genes [47].

Challenge test

Challenge testing followed previously established methods [69]. *Vibrio parahaemolyticus* was activated and cultured in 2216E liquid medium. The LD50 was determined based on pre-test results (LD50:1×10^8 CFU/mL). After the breeding trials, 10 shrimp were randomly selected from each replicate for the challenge test. Subsequently, 100 μL of the bacterial suspension was injected into the muscles of the second and third dorsal segments

Table 3 Primers used in this experiment for quantitative RT-PCR

Genes	Forward (5'-3')	Reverse (5'-3')	GenBank no.
β-actin	TGGACTTCGAGCAGGAGATG	GGAATGAGGGCTGGAACAGG	XM_027364954.1
Dorsal	TTGCGACCACCAGACAAGAG	GCAAGGTAACGACTAATCTTCTCTG	XM_027382195.1
Relish	CTACATTCTGCCCTTGACTCTGG	GGCTGGCAAGTCGTTCTCG	XM_027357254.1
PO	TACATGCACCAGCAAATTATCG	AGTTTGGGGAAGTAGCCGTC	XM_027379995.1
ALF	TTACTTCAATGGCAGGATGTGG	GTCCTCCGTGATGAGATTACTCTG	KJ000049
Pen-3	CACCCTTCGTGAGACCTTTG	AATATCCCTTTCCCACGTGAC	Y14926
Crus	CCACGAACCAGAGACACCTG	CGAGGCCAGCACACTTGTAG	AY486426
TOR	GCAGATCCTTGAGAAGACGC	CTGACAGCCGCATTGAGGTA	[26]
4E-BP	ATGTCTGCTTCGCCCGTCGCTCGCC	GGTTCTTGGGTGGGCTCTT	[26]
eIF4E1a	TCCCTTTCCCTAACCCTCA	GTTTTGCTGTCTCGCTTCC	[26]
IGF1	GTGGGCAGGGACCAAATC	TCAGTTACCACCAGCGATT	[41]
IGF2	CTCTGTACAGTCAGCCCAGC	CACACCCAGTCAGTCCCAAG	[41]

PO Phenoloxidase, *ALF* Anti-lipopolysaccharide factor, *Crus* Crustins, *Pen-3* Penaeidin-3a, *TOR* Target of Rapamycin, *4E-BP* eukaryotic translation initiation factor 4E-binding protein, *eIF4E1a* eukaryotic translation initiation factor 4E type 1-Alpha, *IGF1* Insulin-like growth factor 1, *IGF2* Insulin-like growth factor 2

of each shrimp, and the number of deceased shrimp was recorded every 4 h after injection.

Statistical analysis

SPSS 23.0 (IBM, USA) was used to analyze the experimental data. The CON group was subjected to Dunnett's test separately with the CPC replacement group (CB 0, CB 0.065, CB 0.26, CB 1.04, and CB 4.16). Experimental data were expressed as "mean ± SEM (n = 3)". The intestinal microbiota sequencing results were analysed through the Omicsmart dynamic online analysis platform (http://www.omicsmart. com). Challenge test cumulative survival was inspected on Graphpad prism software (version 10.1.2) using the Gehan Breslow-Wilcoxon method. Mantel tests were plotted using the linkET package in the R 4.3.2 software.

Results

Growth performance of *Litopenaeus vannamei*

Effects of freeze-dried CB powder on the growth performance in *Litopenaeus vannamei* are illustrated in Fig. 1. The FBW, WGR, and SGR were significantly decreased in the CB 0 group ($P < 0.05$) and peaked in the CB 1.04 group (Fig. 1B, C, F; $P > 0.05$). This indicates that high levels of CPC without probiotic supplementation can negatively affect shrimp growth. However, the inclusion of freeze-dried CB powder mitigated these adverse effects, with the CB 1.04% group showing the highest FBW, WGR, and SGR among all groups, although not significantly different from the CON group ($P > 0.05$). FCR was significantly higher in the CB 0 group than in the CON group (Fig. 1D; $P > 0.01$). There were no remarkable differences in SR between the groups (Fig. 1E; $P > 0.05$). Binary regression analysis of SGR indicated that the optimal additive level was 2.11% (9.71×10^9 CFU/kg).

Whole-body composition of *Litopenaeus vannamei*

Effects of freeze-dried CB powder on the whole-body composition of *Litopenaeus vannamei* are presented in Table 4. No significant differences were observed in crude protein, crude lipid, ash, and moisture content between the experimental groups and the CON group ($P > 0.05$). This suggests that replacing 40% of fishmeal protein with CPC, even when supplemented with varying levels of freeze-dried CB powder, did not adversely impact the basic nutritional composition of the shrimp.

Antioxidant and immune enzyme activity of *Litopenaeus vannamei*

The effects of freeze-dried CB powder on the antioxidant and immune enzyme activities of serum in *Litopenaeus vannamei* are summarized in Table 5. Serum SOD activity was significantly increased in the CB 1.04 group ($P < 0.05$), indicating improved oxidative stress defense. GPX activity was remarkably lower in the CB0 group ($P < 0.05$) and highest in the CB 1.04 group ($P > 0.05$). MDA activity decreased in the CB 0.065, CB 1.04, and CB 4.16 groups ($P < 0.05$), further indicating that CB supplementation reduces oxidative stress. Serum T-AOC and ACP activity did not significantly differ in the replacement groups compared to the CON group ($P > 0.05$), suggesting that these parameters were not markedly affected by the dietary treatments. Serum AKP activity was notably decreased in the CB 4.16 group ($P < 0.05$). Interestingly, AKP activity was significantly lower in the CB 4.16% group ($P < 0.05$), which may imply that excessively

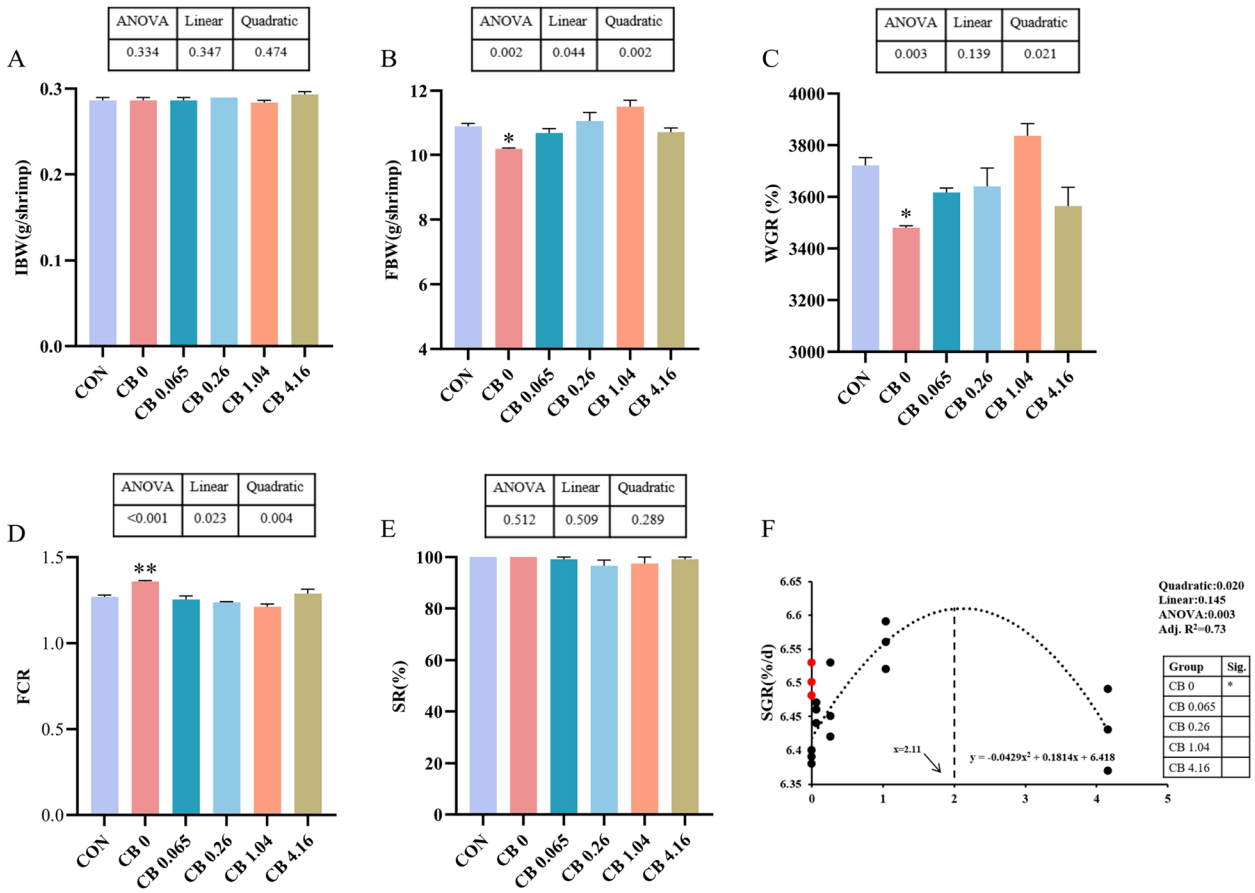

Fig. 1 Effects of freeze-dried CB powder on the growth performance in *Litopenaeus vannamei*. Data are expressed as mean ± SEM ($n = 3$). 40 shrimp per replicate ($n = 40$). * indicates that the values in each group are significantly different from the CON group by the Dennett test. * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$

Table 4 Effects of freeze-dried CB powder on the whole-body composition of *Litopenaeus vannamei*

Items	Treatment						*p* value		
	CON	CB 0	CB 0.065	CB 0.26	CB 1.04	CB 4.16	ANOVA	Linear	Quadratic
Crude protein (%)	72.35 ± 0.25	71.35 ± 0.11	72.07 ± 0.40	72.19 ± 0.49	71.98 ± 0.49	71.70 ± 0.27	0.465	0.631	0.223
Crude lipid (%)	2.58 ± 0.13	1.86 ± 0.08	3.31 ± 0.29	2.79 ± 0.39	2.54 ± 0.32	2.50 ± 0.62	0.195	0.719	0.231
Ash (%)	13.46 ± 0.28	13.48 ± 0.61	13.74 ± 0.06	13.70 ± 0.23	13.78 ± 0.29	13.86 ± 0.49	0.960	0.480	0.774
Moisture (%)	74.91 ± 1.35	74.89 ± 1.60	70.39 ± 1.60	70.07 ± 2.55	72.96 ± 0.20	72.91 ± 2.03	0.261	0.819	0.219

Data are expressed as mean ± SEM ($n = 3$). 40 shrimp per replicate ($n = 40$)

* indicates that the values in each group are significantly different from the CON group by the Dennett test

* indicates $P < 0.05$

** indicates $P < 0.01$

*** indicates $P < 0.001$

high levels of CB could have an inhibitory effect on this enzyme. C3 and C4 activities were lowest in the CB0 group ($P < 0.05$).

Muscle amino acids of *Litopenaeus vannamei*

Muscle amino acid composition of *Litopenaeus vannamei* is shown in Table 6. Threonine content was remarkably higher in the CB 0.065, CB 0.26, and CB 1.04 groups ($P < 0.05$). Methionine content, another vital

Table 5 Effects of freeze-dried CB powder on the activities of antioxidant and immune enzymes of*Litopenaeus Vannamei*

Items	Treatment						*p* value		
	CON	CB 0	CB 0.065	CB 0.26	CB 1.04	CB 4.16	ANOVA	Linear	Quadratic
SOD (U/ml)	94.75 ± 8.11	74.75 ± 4.14	79.78 ± 6.99	119.73 ± 6.55	121.72 ± 4.12*	115.01 ± 6.82	< 0.001	0.001	< 0.001
GPX (U/L)	130.59 ± 7.05	95.38 ± 5.55*	124.31 ± 6.19	127.27 ± 13.54	163.00 ± 4.66	152.31 ± 10.30	0.002	< 0.001	0.001
MDA (mmol/ml)	8.69 ± 0.51	8.67 ± 0.68	6.01 ± 0.58*	7.37 ± 0.97	3.96 ± 0.12**	5.62 ± 0.51*	0.019	0.025	0.065
T-AOC (mmol/L)	0.36 ± 0.01	0.30 ± 0.03	0.32 ± 0.03	0.36 ± 0.01	0.40 ± 0.01	0.33 ± 0.02	0.063	0.112	0.055
ACP (U/100 ml)	26.27 ± 0.43	24.05 ± 0.67	27.92 ± 1.92	29.57 ± 1.45	29.15 ± 1.21	30.98 ± 1.00	0.019	0.003	0.007
AKP (U/100 ml)	9.43 ± 0.45	7.45 ± 0.85	7.63 ± 0.16	8.90 ± 0.13	7.73 ± 0.09	7.09 ± 0.69*	0.036	0.737	0.139
C3 (μg/ml)	73.20 ± 8.86	30.01 ± 2.88**	56.82 ± 2.18	74.11 ± 15.76	89.07 ± 4.98	83.14 ± 1.95	0.002	< 0.001	< 0.001
C4 (μg/ml)	235.96 ± 15.00	164.88 ± 17.24*	194.44 ± 15.68	198.46 ± 17.94	250.32 ± 7.77	195.23 ± 24.54	0.041	0.097	0.075

Data are expressed as mean ± SEM ($n = 3$). 40 shrimps per replicate ($n = 40$)

SOD Superoxide dismutase, *GPX* Glutathione peroxidase, *MDA* Malondialdehyde, *T-AOC* Total antioxidant capacity, *ACP* Acid phosphatase, *AKP* Alkaline phosphatase, *C3* Complement 3, *C4* Complement 4

* indicates that the values in each group are significantly different from the CON group by the Dennett test.

* indicates $P < 0.05$

** indicates $P < 0.01$

**c* indicates $P < 0.001$

Table 6 Effects of freeze-dried CB powder on the muscle amino acids of*Litopenaeus Vannamei*

Items	Treatment						*p* value		
	CON	CB 0	CB 0.065	CB 0.26	CB 1.04	CB 4.16	ANOVA	Linear	Quadratic
EAA (g/100 g)									
Threonine	2.90 ± 0.01	2.93 ± 0.01	3.04 ± 0.02**	3.02 ± 0.03**	2.99 ± 0.03*	2.97 ± 0.03	0.006	0.827	0.037
Valine	3.16 ± 0.01	3.05 ± 0.07	3.12 ± 0.05	2.97 ± 0.08	3.16 ± 0.05	3.17 ± 0.02	0.104	0.177	0.284
Methionine	2.12 ± 0.00	2.19 ± 0.01	2.25 ± 0.01**	2.22 ± 0.02*	2.31 ± 0.03***	2.23 ± 0.02**	< 0.001	0.401	0.638
Isoleucine	3.13 ± 0.02	3.09 ± 0.09	3.17 ± 0.08	3.04 ± 0.08	2.99 ± 0.10	3.19 ± 0.02	0.403	0.930	0.626
Leucine	5.68 ± 0.03	5.82 ± 0.08	5.88 ± 0.08	5.89 ± 0.04	5.96 ± 0.06*	5.97 ± 0.06*	0.044	0.054	0.166
Phenylalanine	3.03 ± 0.01	3.06 ± 0.08	3.06 ± 0.06	3.12 ± 0.11	3.32 ± 0.09*	3.21 ± 0.04	0.089	0.042	0.131
Lysine	6.33 ± 0.02	6.46 ± 0.09	6.48 ± 0.08	6.43 ± 0.03	6.44 ± 0.04	6.41 ± 0.08	0.610	0.419	0.726
Histidine	1.60 ± 0.02	1.58 ± 0.05	1.66 ± 0.03	1.62 ± 0.01	1.63 ± 0.04	1.72 ± 0.02	0.0.084	0.052	0.130
Arginine	8.13 ± 0.03	8.39 ± 0.06*	8.37 ± 0.07*	8.23 ± 0.02	8.22 ± 0.06	8.35 ± 0.04	0.024	0.246	0.086
∑EAA	36.07 ± 0.07	36.58 ± 0.46	37.05 ± 0.32	36.54 ± 0.23	37.04 ± 0.23	37.22 ± 0.26	0.107	0.225	0.447
NEAA (g/100 g)									
Aspartic acid	7.91 ± 0.02	8.09 ± 0.05	8.14 ± 0.06	8.02 ± 0.12	7.99 ± 0.06	8.01 ± 0.07	0.347	0.185	0.429
Serine	2.80 ± 0.02	2.85 ± 0.02	2.91 ± 0.05	2.91 ± 0.03	2.94 ± 0.05	2.98 ± 0.02*	0.052	0.021	0.078
Glutamic acid	13.10 ± 0.10	13.13 ± 0.15	13.23 ± 0.12	13.00 ± 0.10	13.23 ± 0.13	13.33 ± 0.07	0.420	0.306	0.355
Glycine	6.89 ± 0.11	6.72 ± 0.10	6.91 ± 0.05	6.53 ± 0.10	6.45 ± 0.22	6.93 ± 0.12	0.071	0.930	0.356
Alanine	5.13 ± 0.04	5.01 ± 0.26	5.24 ± 0.22	5.07 ± 0.16	4.83 ± 0.10	4.82 ± 0.14	0.453	0.170	0.291
Tyrosine	2.79 ± 0.01	2.86 ± 0.05	2.92 ± 0.05	3.16 ± 0.14*	3.36 ± 0.15**	3.03 ± 0.03	0.007	0.066	0.035
Proline	4.25 ± 0.12	4.53 ± 0.11	4.39 ± 0.01	4.69 ± 0.19	4.93 ± 0.17**	4.54 ± 0.04	0.034	0.267	0.337
∑NEAA	42.89 ± 0.20	43.19 ± 0.28	43.74 ± 0.29*	43.38 ± 0.13	43.73 ± 0.04*	43.63 ± 0.16	0.063	0.212	0.367
∑TAA	78.96 ± 0.13	79.78 ± 0.61	80.79 ± 0.56*	79.93 ± 0.36	80.77 ± 0.20*	80.85 ± 42*	0.040	0.173	0.411

Data are expressed as mean ± SEM ($n = 3$). 40 shrimps per replicate ($n = 40$)

EAA Essential amino acid, *NEAA* Non-essential amino acids, ∑*EAA* Total essential amino acids, ∑*NEAA* Total non-essential amino acids

* indicates that the values in each group are significantly different from the CON group by the Dennett test.

* indicates $P < 0.05$

** indicates $P < 0.01$

*** indicates $P < 0.001$

EAA, was significantly increased in all CB-supplemented groups ($P<0.05$), indicating improved amino acid availability. Leucine levels were significantly elevated in the CB 1.04% and CB 4.16% groups ($P<0.05$), which may contribute to better growth performance due to leucine's role in muscle protein synthesis. The CB 1.04% group exhibited the highest phenylalanine content among all groups ($P<0.05$), further supporting the positive effect of CB on amino acid profiles. The serine content was significantly increased in the CB 1.04 group ($P<0.05$). Tyrosine content was significantly higher in CB 0.26 and CB 1.04 groups ($P<0.05$). Proline content was remarkably increased in the CB 1.04 group than in the CON group ($P<0.01$). Total non-essential amino acid content was notably increased in the CB 0.065 and CB1.04 groups than in the CON group ($P<0.05$). Total amino acid content was remarkably higher in the CB 0.065, CB1.04, and CB4.16 groups ($P<0.05$). This improvement in amino acid composition may contribute to better meat quality and nutritional value of the shrimp, making freeze-dried CB powder supplementation beneficial not only for growth but also for meat quality.

Intestinal digestive enzyme activity

The CB 1.04% group exhibited the highest trypsin activity among all groups ($P<0.001$), indicating enhanced protein digestion capacity (Table 7). Amylase activity was significantly decreased in the CB0 group than in the CON group, whereas it was significantly increased in all freeze-dried CB powder-added groups ($P<0.05$). Amylase activity, responsible for carbohydrate digestion, was significantly reduced in the CB 0 group compared to the CON group, reflecting impaired starch digestion due to CPC inclusion without CB. However, all CB-supplemented groups showed a significant increase in amylase activity ($P<0.05$), with levels exceeding the CON group, demonstrating that CB supplementation restores and enhances carbohydrate digestion. Likewise, lipase activity was markedly decreased in the CB0 group ($P<0.01$). Lipase activity was significantly increased in the CB 1.04 and CB 4.16 groups than in the CON group ($P<0.01$). These results suggest that freeze-dried CB powder supplementation improves the overall digestive capacity of shrimp by enhancing the activities of key digestive enzymes, thereby promoting better nutrient utilization and growth performance.

Intestinal short-chain fatty acids (SCFAs) content of *Litopenaeus vannamei*

As shown in Table 8, acetic acid content was remarkably higher in CB 0.26 group ($P<0.01$). Propionic acid content was remarkably increased in CB 1.04 and CB 4.16 groups ($P<0.001$). Conversely, butyric acid content was significantly lower in the CB0 group ($P<0.05$), highlighting that the absence of CB supplementation in diets with high CPC negatively affects butyrate production, which is crucial for intestinal health and epithelial integrity. No significant differences were observed in isobutyric and isovaleric acid content compared to the CON group among the replacement groups ($P>0.05$). Notably, with the addition of CB bacterial powder, isovaleric and isobutyric acid content exhibited an initial increase followed by a decrease. Overall, these findings suggest that freeze-dried CB powder supplementation modulates the intestinal SCFAs content, enhancing beneficial SCFAs that support gut health and nutrient absorption in shrimp fed high levels of plant protein.

Table 7 Effects of freeze-dried CB powder on the intestinal digestive enzyme activities of*Litopenaeus Vannamei*

Items	Treatment						*p* value		
	CON	CB 0	CB 0.065	CB 0.26	CB 1.04	CB 4.16	ANOVA	Linear	Quadratic
Trypsin (U/mgProt)	2157.07 ± 299.43	1492.32 ± 137.41	2134.09 ± 122.63	2535.10 ± 134.97	3567.74 ± 127.30***	2117.58 ± 166.75	< 0.001	0.041	0.002
Amylase (mU/mgProt)	491.25 ± 11.88	396.60 ± 36.42*	592.53 ± 26.57*	726.82 ± 17.44***	824.01 ± 3.03***	776.69 ± 25.89***	< 0.001	< 0.001	< 0.001
Lipase (mU/mgProt)	807.09 ± 23.79	542.23 ± 10.18**	809.96 ± 57.57	927.36 ± 32.09	1029.43 ± 65.57**	1156.41 ± 22.50***	< 0.001	< 0.001	< 0.001

Data are expressed as mean ± SEM ($n=3$). 40 shrimps per replicate ($n=40$)

* indicates that the values in each group are significantly different from the CON group by the Dennett test

* indicates $P<0.05$

** indicates $P<0.01$

*** indicates $P<0.001$

Table 8 Effects of freeze-dried CB powder on the intestinal SCFAs content of*Litopenaeus vannamei*

Items (μg/g)	Treatment						*p* value		
	CON	CB 0	CB 0.065	CB 0.26	CB 1.04	CB 4.16	ANOVA	Linear	Quadratic
Acetic acid	4.04 ± 0.59	2.47 ± 0.48	3.33 ± 0.79	2.87 ± 0.32**	8.05 ± 0.06	6.20 ± 1.16	< 0.001	0.002	0.011
Propionic acid	0.51 ± 0.05	0.54 ± 0.03	0.54 ± 0.06	0.37 ± 0.03	2.47 ± 0.43***	2.32 ± 0.24***	< 0.001	0.001	0.001
Isobutyric acid	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.05 ± 0.02	0.522	0.543	0.597
Butyric acid	0.07 ± 0.01	0.03 ± 0.02*	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.034	0.347	0.070
Isovaleric acid	0.15 ± 0.03	0.08 ± 0.02	0.09 ± 0.01	0.12 ± 0.03	0.09 ± 0.03	0.08 ± 0.01	0.256	0.912	0.530

Data are expressed as mean ± SEM ($n = 3$). 40 shrimps per replicate ($n = 40$)

* indicates that the values in each group are significantly different from the CON group by the Dennett test

* indicates $P < 0.05$

** indicates $P < 0.01$

*** indicates $P < 0.001$

Intestine immunity and growth-related gene expression

The intestinal immunity and growth-related gene expression of *Litopenaeus vannamei* are illustrated in Fig. 2. The relative expression of Dorsal, Relish, *IGF1*, and *IGF2* genes was significantly increased in the CB 1.04 group (Fig. 2A, B, G, K; $P < 0.01$). Intestinal *PO*, *TOR*, and *4E-BP* gene relative expression was remarkably increased in the CB 1.04 and CB 4.16 groups (Fig. 2C, G, H; $P < 0.05$). Additionally, the expression levels of antimicrobial peptide (AMP) genes such as *ALF*, *Pen-3*, and *Crus* were significantly upregulated in the CB 0.26%, CB 1.04%, and CB 4.16% groups (Fig. 2D, E, F; $P < 0.05$). The elevated expression of these AMP genes indicates enhanced innate immune defense mechanisms, which could contribute to better disease resistance. The upregulation of *eIF4E1α* in the CB 0.26% and CB 1.04% groups (Fig. 2I; $P < 0.05$) further indicates enhanced protein translation processes. Collectively, these results demonstrate that freeze-dried CB powder supplementation positively influences both immune function and growth by modulating the expression of key genes involved in immune responses and growth regulation.

Analysis of the intestinal microbiota of *Litopenaeus vannamei*

The relative abundance of shrimp intestinal microbiota at the phylum level for each treatment group is shown in Fig. 3A. Proteobacteria, Bacteroidetes, Actinobacteria and Verrucomicrobia were the major microbiota of shrimp in each treatment group. The relative abundance of Proteobacteria and Bacteroidetes did not show significant differences among the groups (Fig. 3a1, a2; $P > 0.05$), indicating that these major phyla were relatively stable despite dietary changes. The relative abundance of Actinobacteria was significantly higher in the CB 1.04 group (Fig. 3a3; $P < 0.001$). The relative abundance of Verrucomicrobia increased and then decreased with the addition of CB bacterial agents and was highest in the CB 0.26 group (Fig. 3a4; $P < 0.05$). This indicates that moderate freeze-dried CB powder supplementation may favor the proliferation of Verrucomicrobia, potentially enhancing gut health. At the genus level, *Vibrio*, *Demequina*, *Ruegeria*, and *Halocynthiibacter* were the major intestinal microbiota (Fig. 3B). The relative abundance of *Vibrio* was significantly higher in the CB 0.065 group (Fig. 3b1; $P < 0.5$) and lowest in the CB 1.04 group ($P > 0.05$). This suggests that higher levels of freeze-dried CB powder supplementation may suppress potentially harmful bacteria like *Vibrio*. In contrast, the abundance of *Demequina*, a genus associated with beneficial effects, was significantly increased in the CB 1.04% group (Fig. 3b1; $P < 0.01$), indicating that CB promotes beneficial microbiota. The relative abundance of *Ruegeria* was reduced in the CB 0.065 and CB 0.26 groups (Fig. 3b3; $P < 0.05$). Relative abundance of *Halocynthiibacter* was remarkably increased in the CB 0.065 and CB 1.04 groups (Fig. 3b4; $P < 0.05$). There was no significant difference in the α diversity index of each treatment group compared to the CON group (Fig. 3C-G; $P < 0.05$). This indicates that freeze-dried CB powder supplementation did not adversely affect the overall microbial diversity in the shrimp intestine. The total number of OTUs in the six treatment groups was 141 (Fig. 4A). The number of unique OTUs in each group was 204 (CON), 61 (CB 0), 102 (CB 0.065), 81 (CB 0.26), 67 (CB 1.04), and 151 (CB 4.16), respectively. In order to better visualise the effect of CB bacterial agents on the intestinal flora of shrimps, changes in the intestinal flora were analysed using PCoA and NMDS (Fig. 4B, C). As can be seen from the PCoA and NMDS graphs, the CB 0.26 and CB 4.16 groups are farther away. Overall, dietary supplementation with freeze-dried CB powder modulated the intestinal microbiota composition of shrimp, promoting beneficial bacteria while suppressing potential pathogens.

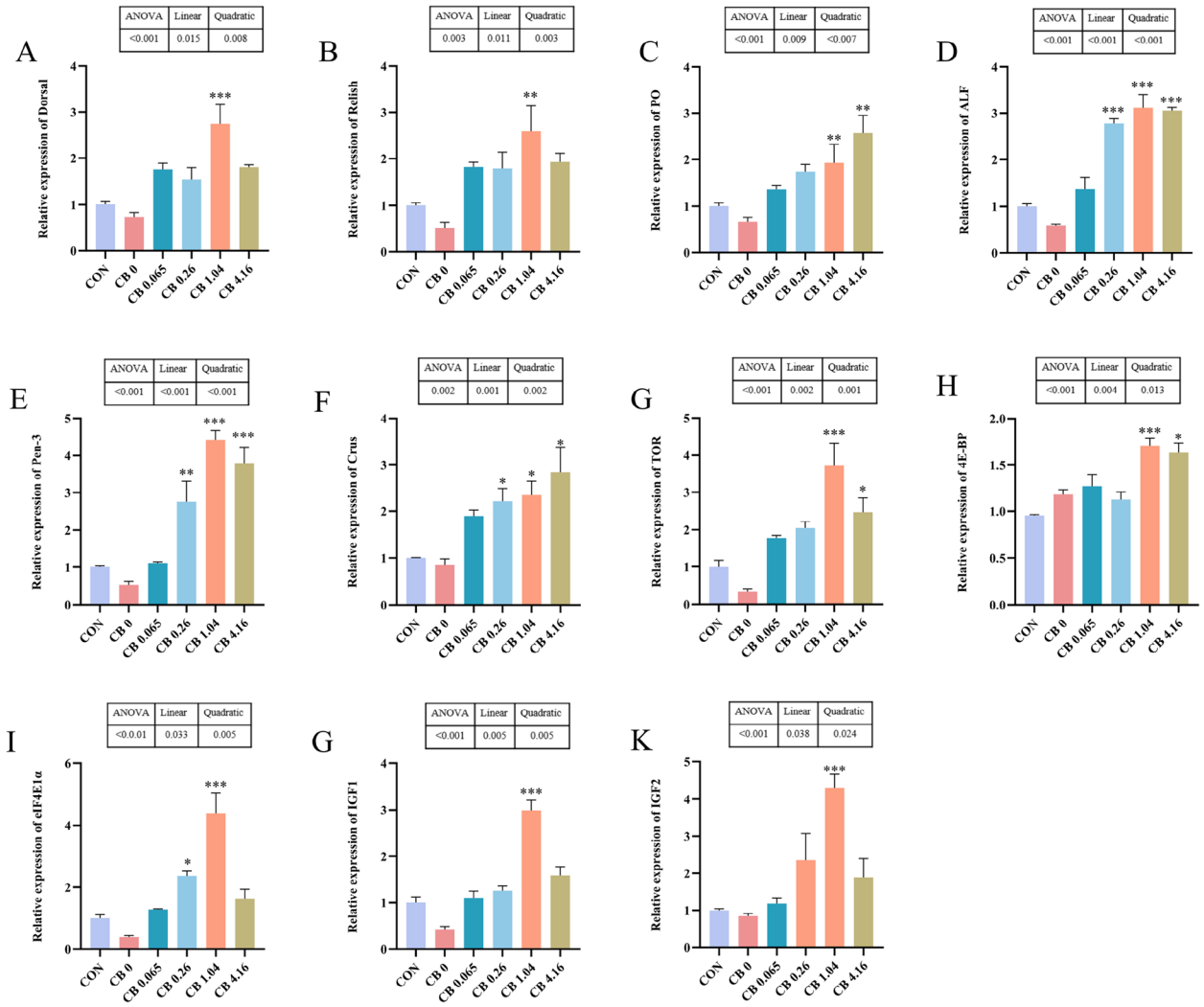

Fig. 2 Effects of freeze-dried CB powder on the immunity and growth-related gene expression in *Litopenaeus vannamei*. Data are expressed as mean ± SEM ($n = 3$). 40 shrimp per replicate ($n = 40$). * indicates that the values in each group are significantly different from the CON group by the Dennett test. * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$

Correlation analysis between intestinal microbiota and physiological indicators

The pearson correlation between the intestinal microbiota and physiological indicators is shown in Fig. 5A. Verrucomicrobia was significantly positively correlated with SOD, SGR, Trypsin, and C4 ($P < 0.05$). This suggests that an increased abundance of Verrucomicrobia is associated with improved growth, enhanced digestion, and a stronger immune response, indicating its beneficial role in shrimp health. Actinobacteria and *Demequina* were remarkably positively correlated with *Pen-3*, *IGF1*, *TOR*, and propionic acid ($P < 0.05$). This implies that these bacteria may contribute to immune modulation, growth promotion, and enhanced energy metabolism through SCFA production. *Vibrio* was significantly negatively correlated with C3 and propionic acid ($P < 0.05$). Bacteroidetes was significantly negatively correlated with WGR and *IGF1*($P < 0.05$). Mantel test analyses of the intestinal microbiota, SCFAs and physiological indices are presented in Fig. 5B. SCFAs were significantly correlated with Dorsal, *TOR*, *IGF2*, and Lipase. At the phylum level, bacterial composition was significantly correlated with *TOR*, *Dorsal*, and *C3* ($P < 0.05$), reinforcing the connection between microbiota and immune-growth regulation. At the genus level, bacteria were significantly correlated with WGR and SOD ($P < 0.05$).

Vibrio parahaemolyticus challenge test

As shown in Fig. 6, the CB 0 group exhibited the highest cumulative mortality rate (43.33%), while the CB1.04 group showed the lowest (20%). However, there

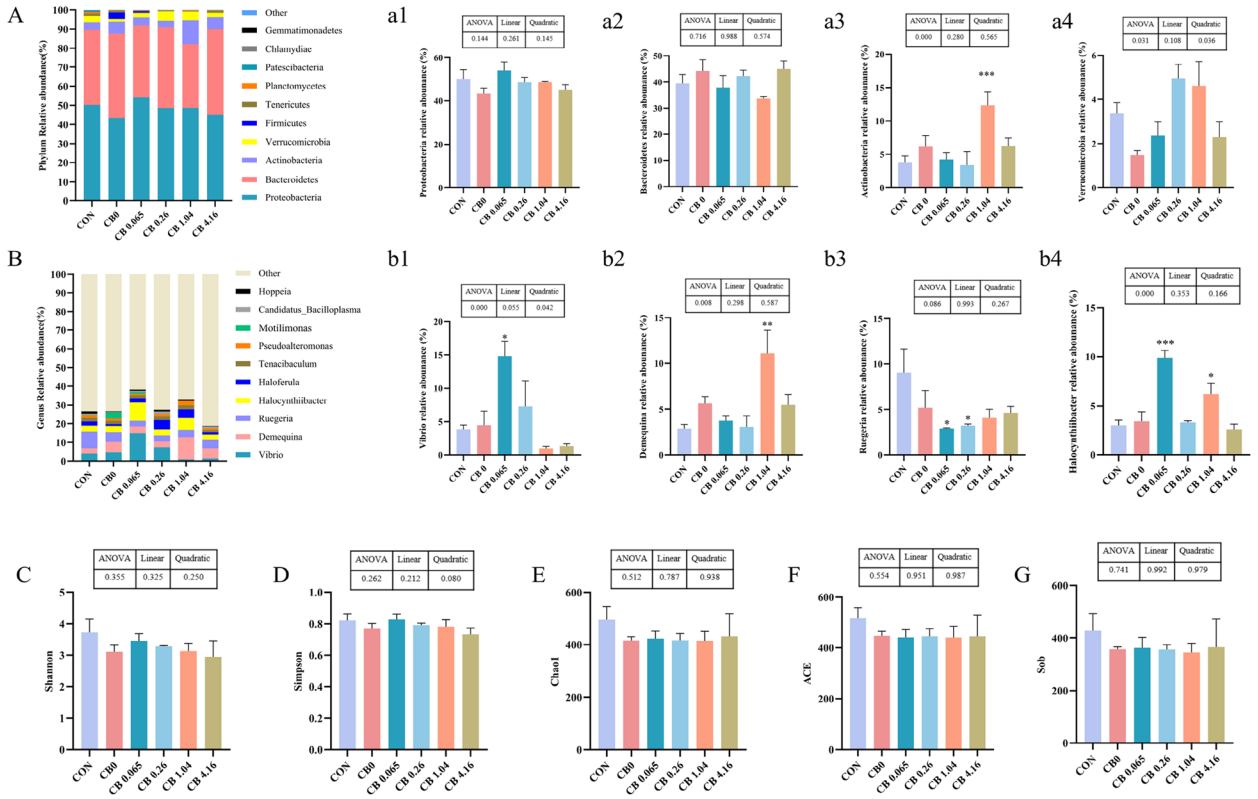

Fig. 3 Effects of freeze-dried CB powder on the intestinal microbiota in *Litopenaeus vannamei*. **A** Top 10 bacteria in relative abundance at the phylum level; **B** Top 10 bacteria in relative abundance at the genus level; a1-a4 (phylum level)/b1-b4 (genus level): Statistical analysis of the top 4 bacteria in relative abundance. Data are expressed as mean ± SEM ($n = 3$). * indicates that the values in each group are significantly different from the CON group by the Dennett test. * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; C-G: Alpha diversity analysis. Data are expressed as mean ± SEM ($n = 3$). 40 shrimp per replicate ($n = 40$). * indicates that the values in each group are significantly different from the CON group by the Dennett test. * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$

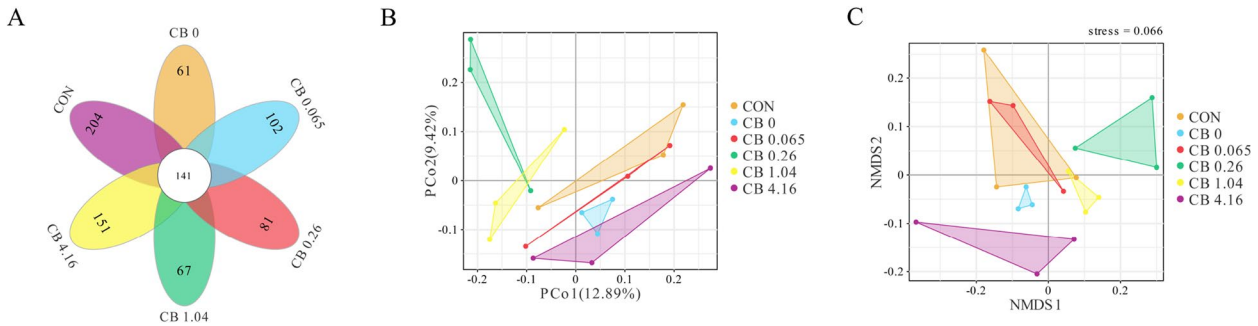

Fig. 4 **A** Venn analysis at the OTU level for each group of samples; **B** PCoA analysis of samples in each group based on OTU level (unweight_unifrac distance); **C** NMDS analysis of samples in each group based on OTU level (unweight_unifrac distance)

was no significant difference in cumulative mortality observed between the gro ups ($P > 0.05$). This suggests that freeze-dried CB powder supplementation strengthens the innate immune system of shrimp, providing better protection against bacterial infections.

Discussion

The growth indices (WGR, SGR) of the CB 0 group in this study were significantly lower than CON group. This is consistent with the results of studies in *Larimichthys crocea* [64] and hybrid grouper [79]. The negative effects

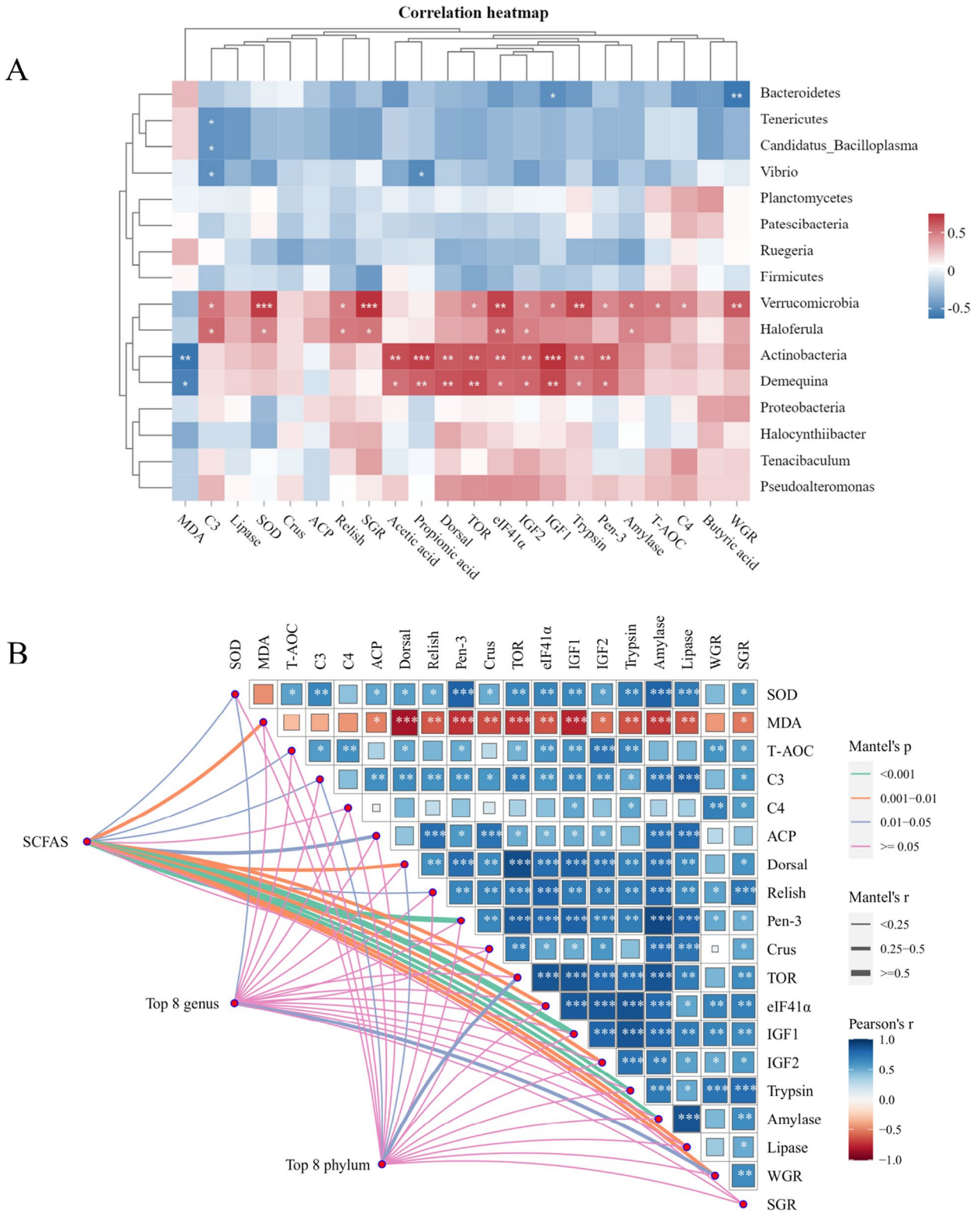

Fig. 5 **A** Pearson correlation analysis of intestinal microbiota with physiological indicators. Blue represents negative correlation and red represents positive correlation. **B** Mantel test analyses of the SCFAs, intestinal microbiota and physiological indices. In the matrix, blue squares signify positive correlations while red squares denote negative correlations. The magnitude of each square reflects the coefficient's value. Lines of various colors outside the matrix show the relationships between gut flora, SCFAs, and physiological indicators

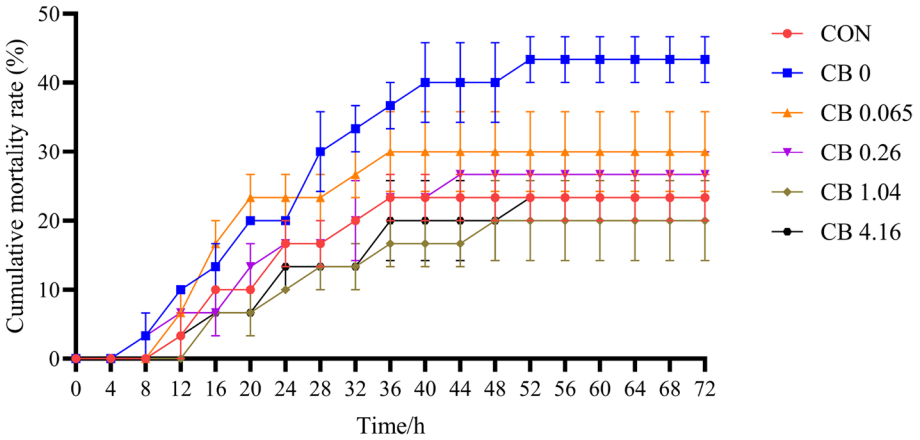

Fig. 6 Effects of freeze-dried CB powder on cumulative mortality after challenge testing in *Litopenaeus vannamei*

of high levels of CPC replacement of fishmeal on aquatic animals are mainly attributed to the anti-nutritional factor, cotton phenol. It was reported that 150 mg/kg of cotton phenol could reduce the intestinal lipase activity in *Oreochromis niloticus*, exacerbate intestinal inflammatory responses and apoptosis, and eventually disrupt intestinal flora [31]. To mitigate these negative effects, the inclusion of functional additives, such as probiotics or prebiotics, may help improve gut health and nutrient absorption. CB are beneficial bacteria found in the intestines of healthy humans and other animals. Compared to other probiotic bacteria such as *Lactobacillus* and *Bacillus*, CB is more tolerant to a wide range of antibiotics and can adapt to higher temperatures and lower pH [21, 35]. The results of the study showed that dietary CB addition improved the growth performance of *Litopenaeus vannamei* [32, 70], *Macrobrachium rosenbergii* [59] and common carp (*Cyprinus carpio* L.) [43]. Dietary CB concentrations of 1×10^4, 1×10^5, 1×10^6, and 1×10^7 CFU/g did not improve the growth performance of *Litopenaeus vannamei*, whereas SGR of *Litopenaeus vannamei* was significantly improved when the concentrations were increased to 1×10^8 and 1×10^9 CFU/g [27]. There was no significant improvement in the growth performance of *Penaeus monodon* when the dietary CB concentration was 5×10^6 CFU/g, whereas it significantly increased FBW and WGR when the dietary CB concentration was 1×10^7 and 2×10^7 CFU/g [10]. Supplementation of 200 mg/g of CB (1×10^9 CFU/g) in *Marsupenaeus japonicus* diets significantly increased FBW and WGR, and significantly decreased FCR [12]. The results of this study showed that the CPC replacement of 40% fishmeal protein reduced the FBW and WGR of shrimp, but improved it with the supplementation of CB bacterial agent. This is consistent with the existing studies [33]. CB supplements insufficient endogenous enzyme activity caused by the external environment by secreting digestive enzymes directly into the animal's gut [42]. These digestive enzymes accelerate the breakdown of indigestible carbohydrates in the diet and promote the absorption of nutrients by the body [7]. In the present study the addition of CB freeze-dried bacterial agent significantly increased intestinal trypsin, amylase and lipase activities in shrimp. Duan et al. have demonstrated that CB can significantly increase intestinal amylase and lipase activities in *Penaeus monodon* [33] and *Marsupenaeus japonicus* [8]. In a study with *Litopenaeus vannamei*, dietary supplementation with CB at 2.5×10^9, 5.0×10^9 and 1.0×10^{10} CFU/kg increased intestinal digestive enzyme activities [9]. The results of studies on CB to enhance the activities of three digestive enzymes were similarly reported in *Macrobrachium rosenbergii* [59], *Lateolabrax maculatus* [23] and hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) [47]. Therefore, CB freeze-dried bacterial agents may improve growth performance by promoting the secretion of digestive enzymes in *Litopenaeus vannamei*. Aquatic animals differ in their ability to absorb and utilise nutrients, and differences in dietary components may lead to changes in whole body composition levels in the organism [78]. In this experiment, CB freeze-dried bacterial agent had no significant effect on whole body composition of shrimp.

SOD serves as a useful indicator for assessing the oxidative stress levels in aquatic organisms by converting superoxide anions generated during microbial invasion into hydrogen peroxide or molecular oxygen, thus establishing an initial defense mechanism against oxidative damage [1, 5]. T-AOC is a biomarker that measures antioxidant potential, which functions to prevent oxidative damage to cell membranes and other cellular components [51]. MDA is a product of lipid peroxidation and is utilized to gauge the degree of cellular injury inflicted by

reactive oxygen species [18]. GPX functions by catalyzing the conversion of glutathione to oxidized glutathione, thereby transforming harmful hydrogen peroxide and lipid peroxides into harmless hydroxyl compounds [74]. In the present study freeze-dried CB powder supplementation increased the activity of serum SOD and GPX, and decreased MDA activity in shrimp. This is consistent with the results of studies in *Macrobrachium rosenbergii* [72]. Addition of 1×10^8 CFU/g CB to the diet increased serum and hepatopancreatic SOD activity in the *Eriocheir sinensis* [15]. Dietary supplementation with 0.5-2.0% CB (1×10^9 CFU/g) significantly increased intestinal SOD and GPX activities in *Penaeus monodon* after nitrite stress [10]. ACP is one of the marker enzymes of lysosomes, while AKP plays a pivotal role as an immunoreactive enzyme involved directly in phosphate group transfer, exhibiting significant regulatory functions [82]. In this experiment, freeze-dried CB powder increased serum ACP, AKP, C3, and C4 activities in shrimp. Our study found significant increases in AKP and ACP activities in shrimp serum following freeze-dried CB powder supplementation, consistent with results in Scophthalmus maximus [3], *Larimichthys crocea* [80], and Lateolabrax maculatus [22], where CB increased these enzyme activities. Complement plays a crucial role in microbial infection defense, exerting various biological effects such as toxin neutralization, inflammation mediation, and pathogenic bacteria clearance [57]. In this study, freeze-dried CB powder significantly increased serum C3 and C4 levels, corroborating findings in *Oreochromis niloticus* [28]. The enhancement of antioxidant and immunity by CB in farmed animals may be related to its metabolites. CB can produce NADPH and H_2, effectively preventing oxidation [85]. Moreover, the absorption of SCFAs produced by CB in the intestinal mucosa regulates immune cell activity, thereby boosting immunity [33].

Amino acids in shrimp muscle are essential components for evaluating the nutritional quality of shrimp, as they play a key role in protein synthesis and muscle growth. The balance of amino acids also influences feed efficiency and the overall physiological function of shrimp, making them critical parameters for aquaculture studies [38]. Furthermore, amino acid composition serves as an important indicator of shrimp meat quality, contributing to its flavor and market value. Amino acids with small hydrophobic side chain such as glycine, alanine, serine, and proline have a sweet flavour, while those with large hydrophobic side chain such as leucine, isoleucine, valine and phenylalanine have a bitter flavour [50]. Studies have shown that CB supplementation improves muscle essential amino acid, flavour amino acid and total amino acid content in finishing goats [84]. Similarly, dietary CB at 400–600 mg/kg significantly increased alanine, aspartic acid, glycine, glutamic acid, and tyrosine content in the breast muscle of Peking ducks [36]. In this study, freeze-dried CB powder supplementation significantly increased serine, tyrosine, and proline content in shrimp muscle, indicating CB's potential to enhance flavor-related amino acids. Threonine is an essential amino acid for a wide range of aquatic animals and is the limiting amino acid for other fish and crustaceans, especially when plant protein sources replace fishmeal as the main protein source in the diet [30, 61]. Leucine, the sole ketogenic amino acid among branched-chain amino acids, plays key roles in haemoglobin synthesis, blood glucose level maintenance, and hormone production [13]. In this experiment, supplementation CPC diet with freeze-dried CB powder increased the threonine and leucine content in shrimp muscle. Further research is needed to elucidate the exact mechanisms by which CB improves muscle amino acid composition and quality in *Litopenaeus vannamei*.

CB, a commensal bacterium, utilizes dietary fiber to produce SCFAs, notably butyric and acetic acids, in the intestines of humans and animals. Butyrate, generated via the butyric kinase pathway, serves as a primary energy source for colon gland cells, crucial for maintaining intestinal homeostasis [69]. In this study, freeze-dried CB powder significantly enhanced the content of butyrate in shrimp, a finding consistent with previous research on other aquatic species. For instance, in common carp, dietary CB at concentrations of 0.25×10^7 and 1.0×10^7 CFU/g increased butyric acid levels in the intestines [43]. Similarly, in *Marsupenaeus japonicus*, CB supplementation (100–200 mg/g) significantly increased intestinal butyric acid levels [9]. Acetate is the major metabolite produced by most colonic anaerobic bacteria by fermentation of undigested and unabsorbed carbohydrates in the small intestine. In the present study, shrimp in the CB 0.26 group showed increased acetate levels, aligning with studies in weaned piglets where 2.5×10^9 CFU/kg CB enhanced acetate content [16]. Propionate, absorbed in the colon and metabolized by hepatocytes in the liver, participates in gluconeogenesis. Our results showed a significant increase in propionate levels in the CB 1.04 and CB 4.16 groups, consistent with prior findings in *Litopenaeus vannamei*, where CB improved propionate levels in the intestines [11]. Pearson correlation analysis demonstrated that butyrate, acetate, and propionate were positively correlated with beneficial bacterial genera such as Actinobacteria and *Demequina*, suggesting that these microbial communities are involved in SCFA production. Furthermore, Mantel test analysis revealed a significant positive correlation between SCFAs and key physiological indicators, such as antioxidant enzyme activity and immune response markers, except for C4 and

Crus. These results underscore the role of CB in promoting intestinal homeostasis and enhancing shrimp health through the production of SCFAs.

Shrimp rely on their innate immune system to defend against pathogenic infections, which includes cellular and humoral immunity. Humoral immunity in shrimp consists mainly of the phenoloxidase system, the coagulation cascade reaction, and antimicrobial peptide synthesis and secretion mediated by innate immune signalling pathways [49]. *ALF*, *Pen-3*, and *Crus* are common antimicrobial peptide genes. The *ALF* gene plays a critical role in innate immunity by directly killing pathogens or activating the host immune system, thereby enhancing the host's defense against pathogens [58]. *Crus* represents a class of cationic antimicrobial peptides that are rich in cysteine and were among the first to be discovered and widely distributed in crustaceans, participating in antimicrobial immune responses [20]. The penaeidin family constitutes an important class of antimicrobial effector molecules. Among these, *Pen-3* is the most abundant peptide in the *Litopenaeus vannamei* [71]. The production of antimicrobial peptides is regulated by innate immune signaling pathways, with the Toll pathway and the immunodeficiency pathway being the major ones associated with antimicrobial peptide synthesis in shrimp [19]. Following recognition of pathogen-associated molecular patterns by Toll receptors, through a series of signalling transductions, the transcription factor Dorsa is phosphorylated, and then Dorsal enters the nucleus to initiate the expression of antimicrobial peptide genes [26]. CB has been confirmed to increase the expression of *Crus* genes in the intestine of *Litopenaeus vannamei* [9], this is consistent with the results of this study. A previous study found that supplementing the diet with 125–500 mg/kg of CB (2×10^7 cfu/g) enhanced the intestinal immune response of the giant freshwater prawn (*Macrobrachium rosenbergii*) under ammonia stress by upregulating the relative expression of Dorsal and Toll genes in the intestine [60]. This result was verified in the present study. Li et al. [29] demonstrated that CB improves the relative expression of *Toll*, *IMD*, and Relish genes in the lymphoid organs of *Litopenaeus vannamei* [29]. Our results are consistent with these studies, confirming that CB supplementation can enhance immune gene expression in shrimp. Furthermore, we investigated the mTOR signaling pathway, a critical regulator of cell growth, protein synthesis, and nutrient metabolism. Target of rapamycin (TOR) triggers translation and boosts protein synthesis through ribosomal protein S6 kinase polypeptide 1 and eukaryotic translation initiation factor 4E-binding protein [55]. *eIF4E1α* is crucial for cell growth and is closely linked to immunity in processes downstream of mTORC1 [25]. In this study, the CB 1.04% group exhibited the highest relative expression levels of *TOR*, *4E-BP*, and *eIF4E1α*, suggesting that CB supplementation activates the mTOR pathway to enhance protein synthesis and cell growth. These findings align with those of Li et al. [29], who observed similar upregulation of mTOR pathway genes with CB supplementation [29]. Growth hormone (GH) modulates a variety of behaviours such as growth, immunity, metabolism and osmoregulation in the body. Its growth-promoting effects are mediated directly or indirectly by insulin-like growth factors (*IGF-1* and *IGF-2*), which act as insulin analogs and promote cell differentiation and proliferation [17]. Our results showed that the relative expression of IGF-1 and IGF-2 was markedly increased in the CB 1.04% group, indicating that CB may enhance growth performance by stimulating the GH/IGF axis. This enhancement of growth-related gene expression further supports the positive impact of CB on shrimp health and development.

Probiotics primarily regulate gut microbes, promoting beneficial bacteria growth while inhibiting harmful bacteria proliferation, thus enhancing intestinal flora balance [65, 68]. This balanced flora offers various benefits, including immunity enhancement, reduced risk of intestinal disorders, and improved nutrient absorption [31]. In this study bacteria dominated by Proteobacteria, Bacteroidetes, Actinobacteria and Verrucomicrobia were formed at the phylum level. Statistical analysis revealed the highest relative abundance of Actinobacteria and Verrucomicrobia in the CB 1.04 group. Actinomycetes are commonly found in soil, freshwater, and marine organisms, play a crucial role in the decomposition of organic matter like cellulose and chitin, thereby influencing organic matter turnover and the carbon cycle [40]. In our study, the increased abundance of Actinobacteria in the CB 1.04% group was significantly positively correlated with physiological indicators such as SCFAs, IGF1, and TOR, suggesting a potential role in enhancing growth and immune responses. Verrucomicrobia, present in the inner layer of the intestinal mucosa and abundant in healthy individuals, break down polysaccharides such as mucopolysaccharides and cellulose to provide energy and nutrients [46]. A Pearson correlation analysis revealed that Verrucomicrobia was significantly positively correlated with most physiological indicators. Many actinobacteria can produce antibiotics, such as streptomycin produced by Streptomyces, which is crucial for the development of antimicrobial drugs in medicine [2]. Actinobacteria can produce various secondary metabolites, including enzymes, vitamins, and antitumor compounds, which are important for both ecosystems and industrial production [53]. The increase in the abundance of Actinobacteria may be attributed to the metabolic byproducts of *Clostridium butyricum*,

such as butyric acid, which have the capability to regulate the pH levels of the gut. This regulation makes the intestinal environment more conducive to the growth of beneficial bacteria. A low pH environment helps inhibit the growth of harmful bacteria while promoting the colonization and proliferation of beneficial bacteria, including those within the Actinobacteria. A previous study by Duan et al. [10] demonstrated that Proteobacteria, Bacteroidetes, and Firmicutes predominated at the intestinal phylum level after CB addition to *Litopenaeus vannamei* diet [9]. Another study showed that dietary CB addition significantly increased the abundance of Firmicutes in the intestine of the Eriocheir sinensis [15]. The increase of Actinobacteria and Verrucomicrobia in this study may be attributed to the metabolic byproducts of CB, such as butyric acid, which can regulate gut pH levels, creating a more favorable environment for beneficial bacteria while inhibiting harmful ones. At the genus level the bacteria are dominated by *Vibrio*, *Demequina*, *Ruegeria*, and *Halocynthiibacter*. The relative abundance of *Vibrio* decreased to a minimum in the CB1.04 group, consistent with findings in *Litopenaeus vannamei* [9, 69], mud crabs (*Scylla paramamosain*) [37], and common carp [43]. Previous studies have shown that *Vibrio* abundance in the intestine of shrimp fed diets with a CB concentration of 1×10^7 CFU/g for 30 days was significantly reduced [69]. Yin et al. [80] have demonstrated that dietary CB supplementation reduces potentially pathogenic bacteria in *Larimichthys crocea* intestine [80]. CB has also been shown to reduce the relative abundance of intestinal Aeromonas in *Oreochromis niloticus* [29]. *Demequina*, as a starch-degrading bacterium, can break down complex organic substances, enhancing the digestive function of the intestine and improving the efficiency of nutrient absorption [14]. In the present study, the relative abundance of *Demequina* was significantly increased in the CB1.04 group. Furthermore, Pearson correlation analysis indicated that *Demequina* was positively correlated with most physiological indicators. Studies suggest that higher alpha diversity of intestinal microbiota indicates healthier intestinal flora, with decreased diversity linked to dysbiosis and negative effects on intestinal health [56, 64]. The results of this study showed no significant difference in α-diversity index between the groups, consistent with findings in *Litopenaeus vannamei* [9]. The improvement in intestinal microbiota with freeze-dried CB powder may be related to the short-chain fatty acids it produces.

In aquaculture research, mortality rate serves as a key indicator for assessing the disease resistance of aquaculture subjects. Li et al. [29] showed that CB significantly increased the survival rate of *Litopenaeus vannamei* following 14 days of challenge testing [29]. Dietary CB supplementation increased survival of *Litopenaeus vannamei* in a *Vibrio alginolyticus* challenge test [69]. CB significantly increased the survival rate of mud crabs infected with *Vibrio parahaemolyticus* [37]. Similar results were observed in studies in *Macrobrachium rosenbergii* [59] and *Miichthys miiuy* [48]. Supplementation with freeze-dried CB powder effectively reduces the mortality rate of *Litopenaeus vannamei* infected with *Vibrio* parahaemolyticus, aligning with previous studies that highlight CB's role in enhancing disease resistance in aquaculture species.

This study shows that freeze-dried CB powder supplementation improves *Litopenaeus vannamei* health by modulating the gut microbiota, increasing beneficial bacteria like Actinobacteria and Verrucomicrobia, and enhancing immune responses and growth performance. The reduction in mortality following the *Vibrio parahaemolyticus* challenge underscores CB's role in boosting disease resistance in shrimp. For the shrimp feed industry, these findings underscore the potential of CB as a functional feed additive. CB not only enhances shrimp health by modulating gut microbiota and immune responses but also promotes growth and reduces disease-related losses. This aligns with the industry's goals of improving feed efficiency, reducing the use of antibiotics, and promoting sustainable aquaculture practices.

Conclusion

This study confirmed that freeze-dried CB powder can alleviate the negative effects of CPC replacement of fish meal protein in *Litopenaeus vannamei*. Supplementation of CPC diets with freeze-dried CB powder improves antioxidant and disease resistance, enhances the structure of intestinal flora, increases the content of intestinal short-chain fatty acids and improves intestinal health, thereby enhancing nutrient utilisation and the growth performance of *Litopenaeus vannamei*. Under the conditions of this study, the appropriate additive measure of freeze-dried CB powder was 2.11% (9.71×10^9 CFU/kg).

Abbreviations

FBW	Final body weight
SGR	Specific growth rate
WGR	Weight gain rate
FCR	Feed conversion ratio
SR	Survival rate
ACP	Acid phosphatase
AKP	Alkaline phosphatase
T-AOC	Total antioxidant capacity
MDA	Malondialdehyde
GPX	Glutathione peroxidase
C3	Complement 3
C4	Complement 4
SOD	Superoxide dismutase
PO	Phenoloxidase
ALF	Anti-lipopolysaccharide factor
Crus	Crustins
Pen-3	Penaeidin-3a
TOR	Target of rapamycin

4E-BP	eukaryotic translation initiation factor 4E-binding protein
eIF4E1α	eukaryotic translation initiation factor 4E type 1-alpha
IGF1	Insulin-like growth factor 1
IGF2	Insulin-like growth factor 2

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
Qi Wang: Performed experiments, analysed data and wrote the manuscript. Hongyu Liu: Study design, project management, and manuscript revision. Depeng Fan: Analysis of participation results. Yadong Hu: Sample index testing. Beiping Tan: Purchase of reagents. Shiwei Xie and Qiang Chen: revision of manuscripts.

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Data availability
Data will be made available on request.

Declarations

Ethics approval and consent to participate
This experiment was approved by the Ethics Committee for Animal Experiments of Guangdong Ocean University (Ethics approval No. IACUC-GDOU-10/2019-A0186).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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