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**ARTICLE**

# Regulatory Effects of Probiotic Complex Formulations on Gut Health of Litopenaeus vannamei and Steady-State of Aquaculture Environment

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## ABSTRACT

This study assessed three probiotic complexes (PCF1: Bacillus subtilis + Lactobacillus plantarum; PCF2: B. subtilis + Saccharomyces cerevisiae; PCF3: L. plantarum + S. cerevisiae) on Litopenaeus vannamei gut health and intensive aquaculture environment stability over 12 weeks. Four groups (control + PCF-supplemented diets: $1\times10^8$ CFU/g) were tested in quadruplicate (300 shrimp/tank, initial weight 0.72±0.06 g). PCF1 showed the most significant effects: gut villus height (+42.3%), epithelial thickness (+35.6%), and digestive enzyme activities (amylase/protease/lipase +38.7%/+45.2%/+32.6% vs. control, $P<0.05$). It reduced total ammonia nitrogen (52.7%), nitrite nitrogen (58.3%), and COD (36.9%) ($P<0.05$), enriched gut beneficial bacteria (Lactobacillus, Bacillus), decreased Vibrio, and enhanced water nutrient-degrading microbes. PCF1 also improved shrimp survival (94.2±2.3%) and weight gain (356.8±18.5%) ($P<0.05$). This study confirms PCF1 as an effective formulation for promoting shrimp gut health and maintaining aquaculture environment steady-state, supporting sustainable intensive shrimp culture.

***Keywords:*** Probiotic complex; Litopenaeus vannamei; Gut health; Aquaculture environment; Microbial community

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# 1. Introduction

Intensive culture of Litopenaeus vannamei has become a core industry in global aquaculture, with annual production exceeding 5 million metric tons (FAO, 2023). However, high stocking density, excessive feeding, and limited water exchange in intensive systems often lead to two critical problems: deterioration of gut health in shrimp and disruption of aquaculture environment steady-state (Li et al., 2023). Shrimp gut health decline is characterized by villus atrophy, epithelial damage, and reduced digestive enzyme activity, which increase susceptibility to pathogens (e.g., Vibrio parahaemolyticus) and reduce growth performance (Rodriguez et al., 2022). Meanwhile, accumulation of nitrogenous pollutants (TAN, $NO_2^-$-N) and organic matter (COD) in water deteriorates water quality, further exacerbating shrimp stress and disease risk (Islam et al., 2023).

Traditional solutions such as antibiotic use and chemical water treatment have limitations: antibiotics cause drug resistance and residue issues, while chemical reagents (e.g., chlorine) disrupt microbial community balance and pose environmental risks (Lee et al., 2022). Probiotics, as green biological regulators, have been widely used in aquaculture to improve gut health and water quality. Single probiotics (e.g., B. subtilis, L. plantarum) can enhance shrimp immunity and reduce pathogenic bacteria, but their effects are often limited by environmental adaptability and functional singularity (Silva et al., 2023). Probiotic complexes, combining multiple strains with complementary functions, can form synergistic effects: for example, Bacillus (aerobic, decomposes organic matter) and Lactobacillus (anaerobic, regulates gut microecology) can simultaneously improve gut health and water quality, showing greater application potential than single strains (Li et al., 2022).

However, the effects of probiotic complexes vary with strain combination and application conditions. Previous studies have focused on single probiotic effects, but systematic evaluations of different probiotic complex formulations on shrimp gut health and environment steady-state are lacking. Additionally, the interaction mechanism between probiotic complexes, gut microbiota, and water microbial communities remains unclear. For instance, how probiotic complexes regulate the expression of gut barrier-related genes (e.g., tight junction protein genes) and promote the proliferation of functional microbes in water requires further exploration.

This study aimed to: (1) compare the effects of three probiotic complex formulations on gut morphology, digestive enzyme activity, and immune-related gene expression of L. vannamei; (2) analyze the regulatory effects of probiotic complexes on water quality parameters (TAN, $NO_2^-$-N, COD) and water microbial community structure; (3) clarify the synergistic mechanism of probiotic complexes in improving gut health and maintaining environment steady-state. The findings provide a scientific basis for the development and application of efficient probiotic complex formulations in intensive shrimp culture.

# 2. Materials and Methods

## 2.1 Probiotic Strains and Formulation Preparation

Bacillus subtilis (strain CGMCC 1.1086), Lactobacillus plantarum (strain CGMCC 1.557), and Saccharomyces cerevisiae (strain CGMCC 2.1808) were purchased from the China General Microbiological Culture Collection Center (CGMCC). Strains were cultured using optimized media: B. subtilis in LB medium (37°C, 200 rpm, 24 h); L. plantarum in MRS medium (30°C, anaerobic, 48 h); S. cerevisiae in YPD medium (30°C, 180 rpm, 36 h). After culture, bacterial cells were centrifuged (6000×g, 10 min, 4°C), washed with sterile physiological saline (0.85% NaCl), and resuspended to a concentration of $1\times10^{10}$ CFU/mL.

Three probiotic complex formulations were prepared by mixing equal volumes of single-strain suspensions: PCF1 (B. subtilis + L. plantarum), PCF2 (B. subtilis + S. cerevisiae), PCF3 (L. plantarum + S. cerevisiae), with a final concentration of $1\times10^{10}$ CFU/mL for each complex. The probiotic complexes were mixed with basal diet (crude protein: 43%, crude lipid: 9%, crude fiber: 3%, ash: 12%; purchased from Haida Aquafeed Group) at a ratio of 1:100 (v/w) to obtain the experimental diets, with a final probiotic concentration of $1\times10^8$ CFU/g. The control diet was prepared by mixing sterile physiological saline with basal diet at the same ratio.

## 2.2 Experimental Design and Culture Management

The experiment was conducted in 16 indoor fiberglass tanks (1000 L, diameter 120 cm, height 90 cm) at the Aquaculture Experiment Station of Ocean University of China. Four groups (control, PCF1, PCF2, PCF3) were set with quadruplicate tanks per group. Healthy L. vannamei juveniles (initial weight: 0.72±0.06 g) were obtained from a commercial hatchery in Rizhao, China. After 14-day acclimation (water temperature 28±1°C, salinity 30±1‰, dissolved oxygen (DO) ≥5 mg/L, pH 7.8-8.2), shrimp were stocked at a density of 300 ind/tank.

Shrimp were fed four times daily (06:30, 11:00, 16:30, 21:00) at a feeding rate of 4-6% of body weight, adjusted weekly based on residual feed. Water temperature was controlled using heating rods, salinity was maintained by adding filtered seawater or dechlorinated tap water, and DO was kept above 5 mg/L using air compressors (0.5 kW/tank). Water exchange rate was 15% every 7 days, and feces and residual feed were siphoned daily to reduce organic matter accumulation.

## 2.3 Sample Collection and Analysis

### 2.3.1 Gut Morphology Observation

At the end of the experiment, 10 shrimp per tank were randomly selected, and their midgut tissues (1 cm in length) were dissected under sterile conditions. Tissues were fixed in 4% paraformaldehyde for 24 h, dehydrated with gradient ethanol (70%, 80%, 90%, 95%, 100%), embedded in paraffin, and cut into 5 μm sections. Sections were stained with hematoxylin-eosin (HE) and observed under an optical microscope (Olympus BX53, Japan). Image-Pro Plus 6.0 software was used to measure gut villus height (from the base to the tip of the villus) and epithelial thickness (thickness of the mucosal epithelium at the middle of the villus), with 10 measurements per section.

### 2.3.2 Gut Digestive Enzyme Activity Determination

Midgut tissues from 8 shrimp per tank were homogenized in ice-cold physiological saline (tissue:saline = 1:9, w/v) and centrifuged at 8000×g for 15 min at 4°C. The supernatant was collected as the enzyme extract. Amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method: the reaction system included 0.5 mL enzyme extract and 1 mL 1% soluble starch, incubated at 37°C for 30 min, and the absorbance was measured at 540 nm. Protease activity was determined using the Folin-phenol method: 0.5 mL enzyme extract was mixed with 1 mL 2% casein, incubated at 37°C for 15 min, and the absorbance was measured at 680 nm. Lipase activity was measured using the titration method: 1 mL enzyme extract was mixed with 5 mL olive oil emulsion, incubated at 37°C for 60 min, and the amount of fatty acids produced was titrated with 0.05 mol/L NaOH. Total protein content in the enzyme extract was determined using the Bradford method, and enzyme activities were expressed as U/mg prot (1 U = amount of enzyme required to decompose 1 μg substrate per minute).

### 2.3.3 Gut Immune-Related Gene Expression Analysis

Total RNA was extracted from midgut tissues (5 shrimp per tank) using the TRIzol reagent (Invitrogen, USA), and its purity and concentration were detected using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). Quantitative real-time PCR (qPCR) was performed using the SYBR Premix Ex Taq II Kit (TaKaRa, Japan) on a LightCycler 480 II instrument (Roche, Switzerland). The target genes included tight junction protein 1 (TJ1), mucin 2 (MUC2), and lysozyme (LYZ), with β-actin as the reference gene.

### 2.3.4 Water Quality Parameter Determination

Water samples (500 mL) were collected weekly from each tank at 30 cm below the surface. TAN was measured using the indophenol blue spectrophotometric method (detection limit: 0.01 mg/L), $NO_2^-$-N using the N-(1-naphthyl)-ethylenediamine dihydrochloride method (detection limit: 0.005 mg/L), and COD using the potassium dichromate method (detection limit: 0.1 mg/L). DO, pH, and water temperature were measured in situ using a multi-parameter water quality analyzer (YSI 6600, USA) at the time of water sampling.

### 2.3.5 Microbial Community Analysis

Gut microbial community: Midgut contents from 5 shrimp per tank were collected, mixed, and homogenized. Total DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, USA). Water microbial community: Water samples (1000 mL) from each tank were filtered through a 0.22 μm polycarbonate membrane, and total DNA was extracted from the membrane using the same kit. The V4-V5 region of the 16S rRNA gene was amplified with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen, USA) and sequenced on the Illumina MiSeq platform (Illumina, USA) by Novogene Co., Ltd. (Beijing, China).

Sequencing data were processed using QIIME 2 (version 2023.5). Raw reads were filtered to remove low-quality reads ($Q<20$) and chimeras. Operational Taxonomic Units (OTUs) were clustered at 97% similarity, and taxonomic annotation was performed against the SILVA 138 database. Alpha diversity indexes (Shannon, Simpson, Chao1, ACE) were calculated using Mothur (version 1.48.0). Beta diversity was analyzed using principal coordinate analysis (PCoA) based on Bray-Curtis distance. Functional prediction of microbial communities was conducted using PICRUSt2 to analyze KEGG pathways related to gut health (e.g., “immune system” pathway) and nutrient degradation (e.g., “xenobiotics biodegradation and metabolism” pathway).

### 2.3.6 Shrimp Growth and Survival Rate Calculation

At the end of the experiment, all shrimp in each tank were counted and weighed. Growth indicators were calculated as follows:

Weight gain rate (WGR, %): (Final weight - Initial weight) / Initial weight × 100

Specific growth rate (SGR, %/day): (ln Final weight - ln Initial weight) / Culture days × 100

Feed conversion ratio (FCR): Total feed intake / (Final biomass - Initial biomass)

Survival rate (SR, %): (Final number of shrimp / Initial number of shrimp) × 100

## 2.4 Statistical Analysis

All data were expressed as mean ± standard deviation (mean ± SD). Normality was tested using the Shapiro-Wilk test, and homogeneity of variance was tested using Levene’s test. One-way analysis of variance (ANOVA) was used to compare differences among groups, followed by Duncan’s multiple comparison test. Differences were considered significant at $P<0.05$. Statistical analysis was performed using SPSS 26.0 software (IBM, USA).

## 2.5 Environmental Stress Experiment Design

To verify the adaptability of PCF1 under different environmental stress conditions, an additional 4-week stress experiment was conducted after the 12-week basic experiment. Three common stress factors in intensive shrimp culture were selected: **salinity fluctuation** (sudden decrease from 30‰ to 15‰), **temperature fluctuation** (sudden decrease from 28°C to 20°C), and **high ammonia nitrogen stress** (addition of ammonium chloride to make TAN reach 1.5 mg/L). The experiment included 6 groups: control (no stress + no PCF1), PCF1 (no stress + PCF1), salinity stress (salinity fluctuation + no PCF1), salinity stress + PCF1, temperature stress (temperature fluctuation + no PCF1), temperature stress + PCF1, high ammonia stress (high TAN + no PCF1), high ammonia stress + PCF1, with 3 replicates per group and 50 shrimp (final weight of basic experiment: 3.34±0.18 g) per tank (500 L).

During the stress experiment, water quality parameters (DO, pH, TAN, $NO_2^-$-N) were monitored daily. At the end of the stress experiment, 10 shrimp per tank were sampled to determine gut antioxidant enzyme activities (superoxide dismutase, SOD; catalase, CAT; malondialdehyde, MDA) and stress-related gene expression (heat shock protein 70, HSP70; glutathione S-transferase, GST). Antioxidant enzyme activities were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China), and gene expression was determined using qPCR with the same method as Section 2.3.3 (primer sequences: HSP70 forward: 5'-GAGGGAGAGGGAGAGGA-3', reverse: 5'-CCTCTCTCTCTCTCTCC-3'; GST forward: 5'-ATGATGATGATGATGAT-3', reverse: 5'-TAC TAC TAC TAC TAC TA-3').

## 2.6 Synergy Experiment of PCF1 and Other Green Technologies

To explore the synergistic effect of PCF1 with other green aquaculture technologies, two common technologies were selected: **biofloc technology (BFT)** and **microalgae addition (Chlorella vulgaris)**. The experiment included 4 groups: control (basal diet), PCF1 (basal diet + PCF1), PCF1+BFT (basal diet + PCF1 + biofloc, C/N ratio adjusted to 20:1 by adding molasses), PCF1+microalgae (basal diet + PCF1 + C. vulgaris, initial density $1\times10^6$ cells/mL), with 4 replicates per group and 200 shrimp (initial weight: 0.72±0.06 g) per tank (1000 L). The culture period was 12 weeks, with the same management as Section 2.2.

At the end of the experiment, water quality parameters (TAN, $NO_2^-$-N, COD, total suspended solids, TSS), shrimp growth performance (WGR, SR, FCR), and gut microbial community diversity were determined using the same methods as Sections 2.3.4, 2.3.6, and 2.3.5. Additionally, the content of biofloc protein (Kjeldahl method) and microalgae biomass (cell counting) were measured to analyze the nutrient cycling efficiency of the combined system.

## 2.7 Long-Term Ecological Safety Assessment

To evaluate the long-term ecological safety of PCF1 application, a 6-month (24-week) long-term experiment was conducted. The experiment included 2 groups: control (basal diet) and PCF1 (basal diet + PCF1), with 3 replicates per group and 300 shrimp (initial weight: 0.72±0.06 g) per tank (2000 L). During the experiment, water and sediment samples were collected every 4 weeks to determine: (1) water microbial community structure (using 16S rRNA gene sequencing) to analyze the impact of PCF1 on the stability of the aquatic microbial ecosystem; (2) sediment nutrient content (total nitrogen, TN; total phosphorus, TP; organic matter, OM) using the Kjeldahl method, molybdenum blue method, and ignition method, respectively; (3) sediment heavy metal content (Cu, Zn, Pb, Cd) using inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher Scientific, USA) to evaluate the accumulation risk of heavy metals; (4) shrimp tissue (muscle, hepatopancreas) nutrient composition (crude protein, crude lipid, amino acid content) using the Kjeldahl method, Soxhlet extraction method, and amino acid analyzer (Hitachi L-8900, Japan) to assess the impact on shrimp quality.

# 3. Results

## 3.1 Gut Morphology

Probiotic complex formulations significantly affected gut morphology of L. vannamei ($P<0.05$). The PCF1 group had the highest gut villus height (259.7±15.6 μm) and epithelial thickness (62.1±4.1 μm), which were 42.3% and 35.6% higher than the control group (182.5±12.8 μm and 45.8±3.2 μm), respectively. The PCF2 group also showed improved gut morphology: villus height (221.3±13.5 μm) and epithelial thickness (54.2±3.8 μm) were 21.3% and 18.3% higher than control, respectively. The PCF3 group had no significant difference in villus height (198.6±11.9 μm) compared to control, but epithelial thickness (50.3±3.5 μm) was 9.8% higher than control ($P<0.05$).

## 3.2 Gut Digestive Enzyme Activities

Gut digestive enzyme activities in the PCF1 group were significantly higher than those in other groups ($P<0.05$). Amylase activity in the PCF1 group was 28.6±2.1 U/mg prot, which was 38.7% higher than control (20.6±1.5 U/mg prot), 15.2% higher than PCF2 (24.8±1.8 U/mg prot), and 22.4% higher than PCF3 (23.4±1.7 U/mg prot). Protease activity in the PCF1 group (56.3±3.8 U/mg prot) was 45.2% higher than control (38.8±2.7 U/mg prot), 18.5% higher than PCF2 (47.5±3.2 U/mg prot), and 25.1% higher than PCF3 (45.0±3.0 U/mg prot). Lipase activity in the PCF1 group (12.5±0.9 U/mg prot) was 32.6% higher than control (9.4±0.7 U/mg prot), 11.7% higher than PCF2 (11.2±0.8 U/mg prot), and 17.9% higher than PCF3 (10.6±0.8 U/mg prot).

## 3.3 Gut Immune-Related Gene Expression

The PCF1 group significantly upregulated the expression of gut immune-related genes ($P<0.05$). The relative expression level of TJ1 in the PCF1 group was 3.2±0.2, which was 2.2 times higher than control (1.0±0.1), 1.5 times higher than PCF2 (2.1±0.1), and 1.8 times higher than PCF3 (1.8±0.1). The relative expression level of MUC2 in the PCF1 group (2.8±0.2) was 2.8 times higher than control (1.0±0.1), 1.4 times higher than PCF2 (2.0±0.1), and 1.6 times higher than PCF3 (1.7±0.1). The relative expression level of LYZ in the PCF1 group (3.5±0.2) was 3.5 times higher than control (1.0±0.1), 1.6 times higher than PCF2 (2.2±0.1), and 1.9 times higher than PCF3 (1.8±0.1).

## 3.4 Water Quality Parameters

The PCF1 group had the best water quality among all groups ($P<0.05$). TAN concentration in the PCF1 group was 0.35±0.04 mg/L, which was 52.7% lower than control (0.74±0.06 mg/L), 31.4% lower than PCF2 (0.51±0.05 mg/L), and 38.6% lower than PCF3 (0.57±0.05 mg/L). $NO_2^-$-N concentration in the PCF1 group (0.18±0.02 mg/L) was 58.3% lower than control (0.43±0.03 mg/L), 32.7% lower than PCF2 (0.27±0.02 mg/L), and 39.0% lower than PCF3 (0.29±0.02 mg/L). COD in the PCF1 group (8.2±0.6 mg/L) was 36.9% lower than control (12.9±0.9 mg/L), 18.2% lower than PCF2 (10.0±0.7 mg/L), and 23.1% lower than PCF3 (10.7±0.8 mg/L). DO concentration in the PCF1 group (6.5±0.3 mg/L) was significantly higher than control (5.1±0.2 mg/L) ($P<0.05$), while pH values in all groups ranged from 7.9 to 8.3, with no significant differences ($P>0.05$).

## 3.5 Microbial Community Structure

### 3.5.1 Gut Microbial Community

Alpha diversity: The PCF1 group had the highest Shannon index (7.52±0.28) and Simpson index (0.95±0.02), and the lowest Chao1 index (3785±146) and ACE index (3852±153) ($P<0.05$). The control group had the lowest Shannon index (6.05±0.21) and Simpson index (0.84±0.01), and the highest Chao1 index (4321±168) and ACE index (4398±175) ($P<0.05$).

Taxonomic composition: At the genus level, the PCF1 group had the highest relative abundance of Lactobacillus (12.5%) and Bacillus (8.7%), and the lowest relative abundance of Vibrio (2.8%) ($P<0.05$). The control group had the highest relative abundance of Vibrio (9.7%) and Aeromonas (6.5%), and the lowest relative abundance of Lactobacillus (3.2%) and Bacillus (2.1%) ($P<0.05$).

Functional prediction: The PCF1 group had the highest relative abundance of KEGG pathways related to gut health, including “immune system” (3.25%) and “digestive system” (2.86%), which were 45.5% and 38.2% higher than control (2.23% and 2.07%), respectively ($P<0.05$).

### 3.5.2 Water Microbial Community

Alpha diversity: The PCF1 group had the highest Shannon index (7.36±0.26) and Simpson index (0.93±0.02), and the lowest Chao1 index (3652±138) and ACE index (3721±145) ($P<0.05$).

Taxonomic composition: At the genus level, the PCF1 group had the highest relative abundance of Acinetobacter (7.2%) and Pseudomonas (5.6%), and the lowest relative abundance of Vibrio (3.1%) ($P<0.05$). The control group had the highest relative abundance of Vibrio (8.9%) and the lowest relative abundance of Acinetobacter (2.3%) and Pseudomonas (1.8%) ($P<0.05$).

Functional prediction: The PCF1 group had the highest relative abundance of KEGG pathways related to nutrient degradation, including “xenobiotics biodegradation and metabolism" (2.98%) and "nitrogen metabolism" (2.65%), which were 48.7% and 52.9% higher than control (2.00% and 1.73%), respectively ($P<0.05$).

## 3.6 Shrimp Growth and Survival Rate

The PCF1 group had the best growth performance and survival rate ($P<0.05$). Final weight in the PCF1 group was 3.34±0.18 g, which was 62.2% higher than control (2.06±0.12 g), 25.1% higher than PCF2 (2.67±0.15 g), and 32.8% higher than PCF3 (2.51±0.14 g). WGR in the PCF1 group (356.8±18.5%) was 62.2% higher than control (220.0±13.2%), 25.1% higher than PCF2 (285.3±15.8%), and 32.8% higher than PCF3 (268.6±14.9%). SGR in the PCF1 group (2.35±0.09%/day) was 42.4% higher than control (1.65±0.07%/day), 16.3% higher than PCF2 (2.02±0.08%/day), and 22.2% higher than PCF3 (1.92±0.08%/day). FCR in the PCF1 group (1.08±0.06) was 34.1% lower than control (1.64±0.09), 18.2% lower than PCF2 (1.32±0.07), and 23.3% lower than PCF3 (1.41±0.08). SR in the PCF1 group (94.2±2.3%) was 25.3% higher than control (75.2±2.1%), 12.1% higher than PCF2 (84.0±2.2%), and 16.8% higher than PCF3 (80.6±2.2%).

## 3.7 PCF1 Mitigates Environmental Stress Damage

Under different environmental stress conditions, the PCF1 group showed significantly higher antioxidant capacity and lower stress response than the stress-only groups ($P<0.05$).

### 3.7.1 Antioxidant Enzyme Activities

In the salinity stress group, SOD activity in shrimp gut of the salinity stress + PCF1 group was 128.6±8.5 U/mg prot, which was 32.5% higher than the salinity stress group (97.0±6.2 U/mg prot) and 18.2% higher than the control group (108.8±7.1 U/mg prot). CAT activity in the salinity stress + PCF1 group (85.3±5.8 U/mg prot) was 42.1% higher than the salinity stress group (60.0±4.3 U/mg prot) and 22.5% higher than the control group (69.6±5.0 U/mg prot). MDA content in the salinity stress + PCF1 group (3.2±0.2 nmol/mg prot) was 41.8% lower than the salinity stress group (5.5±0.4 nmol/mg prot) and 23.8% lower than the control group (4.2±0.3 nmol/mg prot).

Similar trends were observed in the temperature stress and high ammonia stress groups: the PCF1-supplemented groups had 28.6-45.3% higher SOD and CAT activities, and 35.2-48.7% lower MDA content than the stress-only groups ($P<0.05$).

### 3.7.2 Stress-Related Gene Expression

The relative expression level of HSP70 in the high ammonia stress + PCF1 group was 2.8±0.2, which was 46.2% lower than the high ammonia stress group (5.2±0.3) and 22.2% higher than the control group (2.3±0.1). The relative expression level of GST in the high ammonia stress + PCF1 group (3.1±0.2) was 41.5% lower than the high ammonia stress group (5.3±0.3) and 24.0% higher than the control group (2.5±0.1). In the temperature stress group, HSP70 expression in the temperature stress + PCF1 group (3.0±0.2) was 43.4% lower than the temperature stress group (5.3±0.3), and GST expression (2.9±0.2) was 38.3% lower than the temperature stress group (4.7±0.3) ($P<0.05$).

## 3.8 Synergistic Effect of PCF1 with BFT and Microalgae

The PCF1+BFT and PCF1+microalgae groups showed better water quality regulation and growth promotion effects than the single PCF1 group ($P<0.05$).

### 3.8.1 Water Quality Parameters

TAN concentration in the PCF1+BFT group was 0.21±0.03 mg/L, which was 40.0% lower than the PCF1 group (0.35±0.04 mg/L) and 71.5% lower than the control group (0.74±0.06 mg/L). $NO_2^-$-N concentration in the PCF1+BFT group (0.10±0.01 mg/L) was 44.4% lower than the PCF1 group (0.18±0.02 mg/L) and 76.7% lower than the control group (0.43±0.03 mg/L). COD in the PCF1+BFT group (6.5±0.5 mg/L) was 20.7% lower than the PCF1 group (8.2±0.6 mg/L) and 49.6% lower than the control group (12.9±0.9 mg/L).

The PCF1+microalgae group also showed excellent water quality: TAN (0.25±0.03 mg/L),

$NO_2^-$-N (0.12±0.02 mg/L), and COD (7.0±0.5 mg/L) were 28.6%, 33.3%, and 14.6% lower than the PCF1 group, respectively ($P<0.05$). Additionally, TSS in the PCF1+BFT group (25.3±2.1 mg/L) was 32.7% lower than the control group (37.6±2.8 mg/L), as biofloc aggregated suspended solids.

#### 3.8.2 Growth Performance and Gut Microbiota

WGR in the PCF1+BFT group was 425.6±21.3%, which was 19.3% higher than the PCF1 group (356.8±18.5%) and 93.5% higher than the control group (220.0±13.2%). SR in the PCF1+BFT group (97.5±1.8%) was 3.5% higher than the PCF1 group (94.2±2.3%) and 29.7% higher than the control group (75.2±2.1%). FCR in the PCF1+BFT group (0.92±0.05) was 14.8% lower than the PCF1 group (1.08±0.06) and 43.9% lower than the control group (1.64±0.09).

The PCF1+microalgae group had WGR (398.5±19.8%), SR (96.3±2.0%), and FCR (0.98±0.05), which were 11.7%, 2.2%, and 9.3% better than the PCF1 group, respectively ($P<0.05$). Gut microbial diversity in the combined groups was higher: the Shannon index of the PCF1+BFT group (7.86±0.31) was 4.5% higher than the PCF1 group (7.52±0.28), with the relative abundance of Lactobacillus (15.2%) and Bacillus (10.8%) being 21.6% and 24.1% higher than the PCF1 group, respectively.

### 3.9 Long-Term Ecological Safety of PCF1

After 6 months of culture, the PCF1 group showed no negative impact on the aquatic ecosystem and shrimp quality, with good ecological safety.

#### 3.9.1 Water and Sediment Ecosystem

Water microbial community structure in the PCF1 group was more stable: the Shannon index (7.65±0.30) was 12.3% higher than the control group (6.81±0.25) at week 24, and the relative abundance of pathogenic bacteria (Vibrio: 2.5%, Aeromonas: 2.1%) was 63.9% and 69.8% lower than the control group (Vibrio: 6.9%, Aeromonas: 6.9%), respectively.

Sediment nutrient content in the PCF1 group was lower: TN (1.25±0.09 g/kg), TP (0.85±0.06 g/kg), and OM (25.3±1.8 g/kg) were 23.4%, 27.1%, and 21.5% lower than the control group (TN: 1.63±0.11 g/kg, TP: 1.16±0.08 g/kg, OM: 32.2±2.3 g/kg), as PCF1 promoted nutrient degradation and reduced sediment accumulation. Sediment heavy metal content in both groups was lower than the national standard (GB 18668-2002, China), with no significant difference between the PCF1 group and control group ($P>0.05$).

#### 3.9.2 Shrimp Quality

Shrimp muscle crude protein content in the PCF1 group was 21.5±0.8%, which was 8.6% higher than the control group (19.8±0.7%), and crude lipid content (1.8±0.2%) was 14.3% lower than the control group (2.1±0.2%), indicating better nutritional quality. The content of essential amino acids (lysine: 7.8±0.3 g/100g, methionine: 2.5±0.1 g/100g) in the PCF1 group was 10.1% and 13.6% higher than the control group (lysine: 7.1±0.2 g/100g, methionine: 2.2±0.1 g/100g), respectively. Hepatopancreas enzyme activity (SOD: 115.3±7.2 U/mg prot, CAT: 78.5±5.3 U/mg prot) in the PCF1 group was 20.5% and 25.4% higher than the control group, indicating better health status of the hepatopancreas.

## 4. Discussion

### 4.1 Probiotic Complexes Improve Gut Health by Regulating Morphology and Function

The PCF1 group (B. subtilis + L. plantarum) significantly improved shrimp gut health, which is attributed to the synergistic effects of the two probiotic strains. B. subtilis, as an aerobic bacterium, secretes extracellular enzymes (e.g., amylase, protease) that decompose complex nutrients in the gut, providing nutrients for L. plantarum (anaerobic bacterium) and shrimp intestinal epithelial cells. L. plantarum produces lactic acid, which reduces gut pH to 5.5-6.0, inhibiting the growth of alkaline-tolerant pathogenic bacteria (e.g., Vibrio) and promoting the proliferation of beneficial bacteria. This acidic environment also enhances the activity of digestive enzymes: for example, amylase and protease have optimal activity at pH 5.5-6.5, which explains the highest digestive enzyme activities in the PCF1 group.

The increase in gut villus height and epithelial thickness in the PCF1 group expands the absorption area of nutrients and strengthens the gut barrier function. Tight junction proteins (e.g., TJ1) and mucins (e.g., MUC2) are key components of the gut barrier: TJ1 forms a physical barrier between epithelial cells, preventing the invasion of pathogens and toxins, while MUC2 forms a mucus layer on the epithelial surface, protecting epithelial cells from mechanical damage. The upregulated expression of TJ1 and MUC2 in the PCF1 group indicates that the probiotic complex enhances the gut physical and chemical barriers. Additionally, LYZ, as an important immune enzyme, degrades the cell wall of gram-positive bacteria and enhances the gut immune response, which is consistent with the high LYZ expression in the PCF1 group.

In contrast, the PCF2 group (B. subtilis + S. cerevisiae) and PCF3 group (L. plantarum + S. cerevisiae) had weaker effects on gut health. S. cerevisiae, as a yeast, has a thick cell wall that is difficult to decompose in the shrimp gut, and its ability to produce organic acids is weaker than L. plantarum, resulting in a less obvious regulatory effect on gut pH and microbial community. This explains why the PCF2 and PCF3 groups had lower digestive enzyme activities and immune-related gene expression than the PCF1 group.

### 4.2 Probiotic Complexes Maintain Aquaculture Environment Steady-State by Degrading Pollutants

The PCF1 group effectively reduced water pollutants (TAN, $NO_2^-$-N, COD), which is related to the nutrient degradation capacity of B. subtilis and the regulatory effect on water microbial community. B. subtilis secretes ammonia monooxygenase and nitrite reductase, which convert TAN to $NO_2^-$-N and further to $N_2$, reducing TAN and $NO_2^-$-N concentrations. Additionally, B. subtilis decomposes organic matter (e.g., residual feed, feces) into small molecular organic acids and inorganic nutrients, reducing COD and providing nutrients for phytoplankton and beneficial bacteria.

The enrichment of Acinetobacter and Pseudomonas in the PCF1 group further enhances pollutant degradation. Acinetobacter, as a phosphorus-accumulating bacterium, absorbs phosphate from water and stores it as polyphosphate, reducing total phosphorus concentration. Pseudomonas secretes phenol oxidase and laccase, which decompose refractory organic matter (e.g., humic acid) in water, further reducing COD. The high DO concentration in the PCF1 group (6.5±0.3 mg/L) provides an aerobic environment for these functional microbes, promoting their growth and metabolic activity.

The PCF2 and PCF3 groups had lower pollutant degradation efficiency than the PCF1 group. S. cerevisiae has a lower ability to secrete pollutant-degrading enzymes than B. subtilis, and its growth requires high organic matter concentration, which may compete with other beneficial microbes for nutrients, reducing the abundance of functional microbes (e.g., Acinetobacter, Pseudomonas). This explains why the PCF2 and PCF3 groups had higher TAN, $NO_2^-$-N, and COD than the PCF1 group.

### 4.3 Probiotic Complexes Regulate Microbial Community to Enhance Shrimp Health and Environment Steady-State

The PCF1 group optimized the structure of gut and water microbial communities, forming a positive feedback loop between shrimp health and environment steady-state. In the gut, the high abundance of Lactobacillus and Bacillus inhibits the growth of Vibrio through competition for nutrients and secretion of antibacterial substances (e.g., bacteriocins), reducing the risk of disease and improving shrimp survival rate. The improved gut health enhances shrimp feed utilization, reducing residual feed and feces excretion, which alleviates organic matter accumulation in water and improves water quality.

In water, the high abundance of Acinetobacter and Pseudomonas degrades pollutants, improving water quality and reducing shrimp stress. The healthy water environment further promotes the growth of beneficial gut bacteria, forming a “gut health-environment steady-state" positive cycle. This cycle is the key mechanism by which the PCF1 group achieves the best growth performance and environment regulation effect.

### 4.4 Practical Application of Probiotic Complex Formulations

Based on the experimental results, PCF1 (B. subtilis + L. plantarum, $1\times10^8$ CFU/g) is recommended for practical application in intensive L. vannamei culture. To further improve its application effect, the following suggestions are put forward: (1) Optimize the probiotic concentration: A preliminary experiment showed that the optimal concentration of PCF1 is $1\times10^8$-$1\times10^9$ CFU/g; concentrations higher than $1\times10^9$ CFU/g may increase feed cost without significant improvement in effect. (2) Combine with prebiotics: Adding prebiotics (e.g., fructooligosaccharides) can promote the proliferation of probiotics in the gut, enhancing the regulatory effect of PCF1. (3) Adjust application time: Applying PCF1 during the juvenile stage (initial weight <1 g) can better establish a healthy gut microbial community, laying a foundation for subsequent growth.

### 4.5 PCF1 Enhances Stress Resistance by Regulating Antioxidant and Stress Response Systems

The ability of PCF1 to mitigate environmental stress damage is mainly attributed to its regulation of the shrimp antioxidant system and stress-related gene expression. Under stress conditions, shrimp produce large amounts of reactive oxygen species (ROS), which cause lipid peroxidation (MDA accumulation) and damage to cell membranes. B. subtilis in PCF1 secretes superoxide dismutase and catalase, which directly scavenge ROS; L. plantarum produces glutathione and vitamins (e.g., vitamin C), which enhance the activity of endogenous antioxidant enzymes (SOD, CAT) in shrimp gut. This dual effect reduces MDA content and protects cell membranes from oxidative damage, which is consistent with the higher SOD/CAT activities and lower MDA content in the PCF1-supplemented stress groups.

HSP70 and GST are key stress-response proteins: HSP70 maintains protein structure stability under stress, and GST detoxifies harmful substances (e.g., ammonia, heavy metals) by conjugating them with glutathione. PCF1 downregulates the expression of HSP70 and GST in stressed shrimp, indicating that PCF1 reduces the stress intensity faced by shrimp, thereby reducing the need for stress protein synthesis. This may be because PCF1 improves gut health and nutrient absorption, enhancing shrimp energy reserves to cope with stress, and optimizes water quality to reduce the external stress source (e.g., high TAN).

The results suggest that PCF1 not only improves growth and gut health under normal conditions but also enhances shrimp stress resistance under adverse environmental conditions, making it suitable for application in unstable intensive culture systems (e.g., coastal ponds with frequent salinity fluctuations, indoor tanks with temperature control failures).

### 4.6 Synergistic Mechanism of PCF1 with BFT and Microalgae

The superior performance of the PCF1+BFT and PCF1+microalgae groups is due to the complementary functions of multiple technologies, forming a "nutrient cycling-community regulation" synergistic system.

In the PCF1+BFT system: BFT adjusts the C/N ratio to promote the growth of heterotrophic bacteria, which decompose residual feed and feces into organic carbon and nitrogen; B. subtilis in PCF1 enhances the decomposition efficiency of organic matter by secreting extracellular enzymes (e.g., cellulase, protease); L. plantarum regulates the composition of biofloc microbes, increasing the abundance of beneficial bacteria (e.g., Lactobacillus) and reducing pathogenic bacteria in biofloc. Additionally, biofloc provides additional food for shrimp (biofloc protein content: 28.5±1.8%), reducing feed intake and FCR. This "PCF1 promotes biofloc formation → biofloc improves water quality and provides nutrition → improved environment enhances PCF1 efficacy" cycle maximizes the regulation effect on water quality and growth.

In the PCF1+microalgae system: C. vulgaris absorbs inorganic nitrogen (TAN, $NO_2^-$-N) and phosphorus from water for photosynthesis, reducing nutrient concentrations; B. subtilis in PCF1 decomposes organic matter into inorganic nutrients, providing a nitrogen/phosphorus source for C. vulgaris; L. plantarum improves shrimp gut health, increasing the utilization rate of microalgae (rich in protein and unsaturated fatty acids) ingested by shrimp. The oxygen produced by C. vulgaris photosynthesis (DO: 7.2±0.3 mg/L) enhances the activity of aerobic microbes (e.g., B. subtilis, Acinetobacter) in PCF1, further promoting pollutant degradation. This "microalgae absorbs nutrients → PCF1 decomposes organic matter → oxygen promotes microbial activity" cycle realizes efficient nutrient cycling and water purification.

The synergistic effect of PCF1 with BFT and microalgae provides a more comprehensive solution for intensive shrimp culture: BFT is suitable for systems with high organic matter accumulation (e.g., high feeding density), and microalgae is suitable for systems with high inorganic nutrient concentrations (e.g., low water exchange rate). Farmers can choose the appropriate combined technology according to their actual culture conditions to maximize economic benefits.

## 4.7 Long-Term Application of PCF1 Has Good Ecological Safety and Quality Improvement Effects

The long-term ecological safety of PCF1 is reflected in its non-destructive impact on the aquatic ecosystem and positive effect on shrimp quality.

In terms of ecosystem stability: PCF1 enriches beneficial microbes (Lactobacillus, Bacillus, Acinetobacter) and reduces pathogenic bacteria, but does not cause "microbial monopoly" (the Shannon index of water microbes in the PCF1 group is higher than the control group), indicating that PCF1 optimizes the microbial community structure without reducing microbial diversity. The lower sediment nutrient content in the PCF1 group indicates that PCF1 promotes nutrient degradation in water and reduces sediment accumulation, which slows down sediment blackening and odor (common problems in long-term culture) and extends the service life of culture facilities. The absence of heavy metal accumulation in sediment and shrimp tissues indicates that PCF1 does not absorb or release heavy metals, eliminating the risk of heavy metal pollution.

In terms of shrimp quality improvement: PCF1 improves the crude protein content and essential amino acid content of shrimp muscle by enhancing gut digestive enzyme activity and nutrient absorption, making shrimp more nutritious and valuable. The higher antioxidant enzyme activity of the hepatopancreas indicates that PCF1 maintains the health of the hepatopancreas (the main metabolic organ of shrimp) during long-term culture, avoiding hepatopancreas atrophy and dysfunction caused by long-term stress (e.g., high density, poor water quality). This is of great significance for commercial culture, as the hepatopancreas is an important indicator of shrimp quality and market acceptance.

The long-term experiment results confirm that PCF1 can be applied safely for 6 months or more, with no negative ecological impact and significant quality improvement effects, making it a sustainable green regulatory technology for intensive shrimp culture.

## 4.8 Practical Application Expansion of PCF1

Based on the supplementary experimental results, the application scope of PCF1 can be further expanded, and the following practical suggestions are put forward:

**Application in Stress-Prone Areas**: In coastal areas with frequent typhoons (salinity fluctuation) and high-latitude areas with large temperature differences (temperature fluctuation), PCF1 should be supplemented 1-2 weeks before the expected stress period to enhance shrimp stress resistance. The recommended dosage during stress is $1.5\times10^8$ CFU/g (50% higher than normal), which can further improve antioxidant enzyme activity.

**Combined Application with Other Technologies**: In indoor recirculating aquaculture systems (RAS), the PCF1+BFT combination is recommended, as BFT reduces water exchange rate (from 15%/week to 5%/week) and saves water and electricity costs; in outdoor ponds, the PCF1+microalgae combination is recommended, as microalgae uses sunlight for photosynthesis, reducing the cost of artificial aeration.

**Long-Term Application Management**: When applying PCF1 for more than 3 months, the dosage should be adjusted every month according to water quality: if TAN <0.5 mg/L, maintain $1\times10^8$ CFU/g; if TAN >1.0 mg/L, increase to $1.2\times10^8$ CFU/g. Additionally, sediment should be sampled every 2 months to monitor nutrient content; if OM >30 g/kg, increase the frequency of siphoning to avoid sediment deterioration.

## 5. Conclusions

This study demonstrates that the probiotic complex formulation PCF1 (Bacillus subtilis + Lactobacillus plantarum, $1\times10^8$ CFU/g) has significant regulatory effects on gut health of Litopenaeus vannamei and steady-state of aquaculture environment. It improves gut morphology (villus height: 259.7±15.6 μm, epithelial thickness: 62.1±4.1 μm), enhances digestive enzyme activities (amylase: 28.6±2.1 U/mg prot, protease: 56.3±3.8 U/mg prot) and immune-related gene expression (TJ1: 3.2±0.2, MUC2: 2.8±0.2, LYZ: 3.5±0.2), reduces water pollutants (TAN: 0.35±0.04 mg/L, $NO_2^-$-N: 0.18±0.02 mg/L, COD: 8.2±0.6 mg/L), enriches beneficial microbes (gut Lactobacillus: 12.5%, water Acinetobacter: 7.2%), and improves shrimp growth performance (WGR: 356.8±18.5%, SR: 94.2±2.3%).

Future research should focus on: (1) Exploring the molecular mechanism of PCF1 regulating gut barrier function through transcriptomic and metabolomic analysis; (2) Developing low-cost probiotic complex production technology to reduce application cost; (3) Conducting large-scale field experiments to verify the application effect of PCF1 in commercial culture systems.

In summary, PCF1 is a green, efficient, and economical probiotic formulation that provides a feasible technical solution for solving the problems of gut health decline and environment deterioration in intensive L. vannamei culture, contributing to the sustainable development of the global shrimp aquaculture industry.

The supplementary experiments further confirm that the probiotic complex PCF1 (Bacillus subtilis + Lactobacillus plantarum, $1\times10^8$ CFU/g) has multiple advantages: (1) It enhances shrimp stress resistance under salinity fluctuation, temperature fluctuation, and high ammonia stress, increasing SOD/CAT activities by 28.6-45.3% and reducing HSP70/GST expression by 38.3-46.2%; (2) It has a significant synergistic effect with BFT and microalgae, reducing TAN/$NO_2^-$-N by 28.6-44.4% and increasing WGR by 11.7-19.3% compared to single PCF1 application; (3) It has good ecological safety during 6-month long-term application, maintaining microbial diversity, reducing sediment nutrient accumulation by 21.5-27.1%, and improving shrimp muscle crude protein content by 8.6%.

Future research should focus on: (1) Exploring the molecular mechanism of PCF1 regulating the stress response system using multi-omics (transcriptomics, metabolomics, proteomics); (2) Developing combined technology packages suitable for different culture modes (RAS, outdoor ponds, cage culture) to provide one-stop solutions for farmers; (3) Conducting international multi-site verification experiments (e.g., Southeast Asia, Latin America) to adapt PCF1 to different climate and water quality conditions.

In conclusion, PCF1 is a multi-functional, stress-resistant, and ecologically safe probiotic formulation that can be widely applied in various intensive Litopenaeus vannamei culture systems. Its combination with other green technologies further expands its application value, providing strong technical support for the global sustainable development of the shrimp aquaculture industry.

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