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ARTICLE

Synergistic Effects of Compound Probiotics (Bacillus + Lactobacillus) and Plant-Derived Extract (CPE) on Growth Performance and Disease Resistance of Litopenaeus vannamei

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ABSTRACT

This study explored the synergistic effects of compound probiotics (CPB: Bacillus subtilis BS-1 + Lactobacillus plantarum LP-2, 2:1) and plant-derived extract (CPE: tea polyphenols + oregano oil, 3:1) on Litopenaeus vannamei. A 12-week experiment (six groups: Control, 0.2% CPB, 0.3% CPE, 0.2%CPB+0.2%CPE, 0.2%CPB+0.3%CPE, 0.02% oxytetracycline (AB); 250 shrimp/tank, initial weight 0.92±0.08 g) was followed by a 7-day Vibrio parahaemolyticus challenge (1×10^7 CFU/mL). The 0.2%CPB+0.3%CPE group performed best: weight gain rate (456.8±26.3%) was 62.3% higher than Control, and superior to CPB, CPE, and AB groups ($P<0.05$). It increased gut villus height by 54.2%, reduced gut Vibrio by 70.2%, and enhanced total hemocyte count (+63.0%), lysozyme (+85.2%), and SOD (+58.6%) vs Control. Challenge survival rate (85.3±3.5%) was 2.5-fold higher than Control and 25.4% higher than AB ($P<0.05$). High-throughput sequencing showed enriched beneficial gut bacteria and upregulated immune pathways. Results confirm the strong synergism of 0.2%CPB+0.3%CPE as an efficient green strategy for sustainable shrimp culture.

Keywords: Compound probiotics; Plant-derived extract; Synergistic effect; Litopenaeus vannamei; Disease resistance; Gut microecology

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1. Introduction

Litopenaeus vannamei is the dominant species in global shrimp aquaculture, but intensive farming faces severe challenges from bacterial diseases (e.g., AHPND caused by *Vibrio parahaemolyticus*) and antibiotic overuse (Liu et al., 2024). Green additives such as probiotics and plant-derived extracts have become alternatives to antibiotics, but single additives have limitations: Probiotics are easily affected by environmental stress (e.g., high temperature, low salinity) leading to low colonization rate, while plant extracts have narrow antimicrobial spectra and potential tissue irritation at high doses (Chen et al., 2023; Martinez et al., 2023).

Compound probiotics (CPB) combining *Bacillus* (aerobic, produces digestive enzymes) and *Lactobacillus* (anaerobic, regulates gut pH) have complementary functions: *Bacillus subtilis* secretes amylase and protease to improve nutrient utilization, while *Lactobacillus plantarum* produces lactic acid to inhibit pathogenic bacteria (Li et al., 2024). Plant-derived extract (CPE) (tea polyphenols + oregano oil) has antioxidant and broad-spectrum antimicrobial activities, but its efficacy is restricted by poor stability in feed processing (Nguyen et al., 2024).

Recent studies suggest that probiotics and plant extracts may have synergistic effects: Probiotics can improve the bioavailability of plant extract components by regulating gut microbial metabolism, while plant extracts can enhance probiotic colonization by inhibiting harmful bacteria and providing nutrients (dos Santos et al., 2024). However, current research on their synergy in shrimp culture is limited to single probiotic + single plant extract combinations, and the optimal ratio, synergistic mechanism, and application conditions remain unclear.

This study aimed to: (1) Determine the optimal combination ratio of CPB and CPE for *L. vannamei*; (2) Clarify the synergistic mechanism on gut health and immune function; (3) Evaluate the synergistic effect on Vibrio resistance compared to antibiotics; (4) Provide a scientific basis for the development of high-efficiency composite green additives.

2. Materials and Methods

2.1 Preparation of Additives

Compound Probiotics (CPB): *Bacillus subtilis* BS-1 (viable count: 1×10^{10} CFU/g) and *Lactobacillus plantarum* LP-2 (viable count: 1×10^{10} CFU/g) were isolated from healthy shrimp gut. They were mixed at 2:1 mass ratio (preliminary experiment confirmed this ratio had the highest gut colonization rate) and added to maltodextrin (carrier) to form CPB powder (viable count: 5×10^{9} CFU/g).

Plant-Derived Extract (CPE): Prepared as described in the previous study (tea polyphenols ≥98%, oregano oil ≥75%, 3:1 mass ratio, powder form).

Composite Additives: CPB and CPE were mixed with basal diet (crude protein: 44%, crude lipid: 10%) to form experimental diets (Table 1).

Table 1. Formulation and nutrient composition of experimental diets (dry matter basis, %)

Ingredient	Control	CPB	CPE	CPB+CPE1	CPB+CPE2	AB
Fish meal	30.0	30.0	30.0	30.0	30.0	30.0
Soybean meal	25.0	25.0	25.0	25.0	25.0	25.0
Wheat flour	20.0	20.0	20.0	20.0	20.0	20.0
Fish oil	5.0	5.0	5.0	5.0	5.0	5.0
Soybean lecithin	2.0	2.0	2.0	2.0	2.0	2.0
Mineral premix	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin premix	2.0	2.0	2.0	2.0	2.0	2.0
CPB (0.2%)	-	0.2	-	0.2	0.2	-
CPE (0.2%/0.3%)	-	-	0.3	0.2	0.3	-
Oxytetracycline	-	-	-	-	-	0.02
Total	100.0	100.0	100.0	100.0	100.0	100.0

2.2 Experimental Design and Culture Management

The experiment was conducted in 24 indoor fiberglass tanks (800 L) at Ocean University of China. Shrimp (initial weight: 0.92±0.08 g) were acclimated for 10 days (water temperature 27±1°C, salinity 28±1‰, DO ≥5 mg/L, pH 7.9-8.3) before stocking (250 ind/tank). Six groups (Control, CPB, CPE, CPB+CPE1, CPB+CPE2, AB) with 4 replicates each were set.

Shrimp were fed 3 times daily (08:00, 14:00, 20:00) at 3-5% body weight, adjusted weekly based on residual feed. Water quality was maintained by daily siphoning (feces/residual feed) and 10% water exchange every 3 days. Temperature was controlled with heating rods, DO with air stones, and pH adjusted with sodium bicarbonate.

2.3 Sample Collection and Analysis

2.3.1 Growth Performance

At the end of the 12-week feeding trial, 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): Feed intake / (Final biomass - Initial biomass)

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

2.3.2 Gut Health Analysis

Gut Morphology: 8 shrimp per tank were sampled. Midgut tissues (0.8 cm) were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5 μm), HE-stained, and observed under a microscope. Villus height (VH) and epithelial thickness (ET) were measured with Image-Pro Plus 6.0.

Digestive Enzyme Activity: Midgut tissues (5 shrimp per tank) were homogenized (1:9, w/v) in physiological saline, centrifuged (8000×g, 15 min, 4°C). Amylase (DNS method), protease (Folin-phenol method), and lipase (titration method) activities were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute).

Gut Microbial Community: Midgut contents (5 shrimp per tank) were mixed. Total DNA was extracted with E.Z.N.A.® Soil DNA Kit. The V4-V5 region of 16S rRNA gene was amplified (primers 515F/907R) and sequenced on Illumina MiSeq platform. Alpha diversity (Shannon, Simpson) and taxonomic composition were analyzed with QIIME 2.

2.3.3 Immune Function Determination

10 shrimp per tank were sampled. Hemolymph (0.5 mL/shrimp) was collected with anticoagulant (0.1 M trisodium citrate, 1:1), centrifuged (800×g, 10 min, 4°C) to separate hemocytes and plasma.

Total Hemocyte Count (THC): Hemocyte suspension was diluted 10-fold and counted with a hemocytometer (×10^4 cells/mL).

Lysozyme (LYZ) Activity: Plasma (50 μL) + *Micrococcus lysodeikticus* suspension (0.3 mg/mL), absorbance measured at 540 nm (U/mL).

Superoxide Dismutase (SOD) Activity: Measured with SOD kit (Nanjing Jiancheng), expressed as U/mL.

Phenoloxidase (PO) Activity: Plasma (100 μL) + L-DOPA (5 mmol/L), absorbance measured at 490 nm (U/mL).

2.3.4 Vibrio Challenge Test

50 shrimp per tank were selected for *V. parahaemolyticus* (AHPND strain) challenge. Bacteria were cultured to 1×10^7 CFU/mL, and shrimp were immersed in bacterial suspension for 2 h. Mortality was recorded daily for 7 days to calculate survival rate. At day 3 post-challenge, 5 shrimp per tank were sampled to measure gut Vibrio abundance (TCBS plate counting).

2.3.5 Immune-Related Gene Expression

5 shrimp per tank were sampled at the end of feeding trial and day 3 post-challenge. Total RNA was extracted from midgut tissues with TRIzol reagent, and cDNA was synthesized with PrimeScript RT Kit. qPCR was performed to detect the expression of TLR1, MyD88, and NF-κB (β-actin as reference gene). Primer

sequences are shown in Table 2.

2.4 Statistical Analysis

Data were expressed as mean ± SD. Normality and homogeneity of variance were tested with Shapiro-Wilk and Levene's tests. One-way ANOVA followed by Duncan's multiple comparison test was used to compare groups. Repeated-measures ANOVA was used for dynamic immune parameters. $P<0.05$ was considered significant. Statistical analysis was performed with SPSS 26.0.

3. Results

3.1 Growth Performance

The CPB+CPE2 group showed the best growth performance ($P<0.05$, Table 3). WGR (456.8±26.3%) was 62.3% higher than Control (281.4±18.5%), 28.5% higher than CPB (355.5±22.1%), 24.7% higher than CPE (366.3±23.4%), and 35.8% higher than AB (336.4±21.7%). FCR (0.98±0.05) was 37.1% lower than Control (1.56±0.08). SR (95.6±2.3%) was 32.8% higher than Control (72.0±2.5%) and 22.7% higher than AB (77.9±2.4%). CPB+CPE1 group (0.2% CPB + 0.2% CPE) had lower WGR (398.7±24.5%) and SR (90.2±2.1%) than CPB+CPE2, indicating CPE dosage affects synergistic efficacy.

3.2 Gut Health

3.2.1 Gut Morphology

CPB+CPE2 group had the most intact gut structure ($P<0.05$, Table 4). Villus height (285.6±16.4 μm) was 54.2% higher than Control (185.2±12.3 μm), 22.8% higher than CPB (232.6±14.5 μm), 18.9% higher than CPE (240.2±15.1 μm), and 29.5% higher than AB (220.5±13.8 μm). Epithelial thickness (65.8±4.2 μm) was 48.6% higher than Control (44.3±2.9 μm), indicating the composite additive promotes gut tissue development.

3.2.2 Digestive Enzyme Activity

Digestive enzyme activities in CPB+CPE2 were significantly higher than other groups ($P<0.05$, Table 5). Amylase (38.5±2.7 U/mg prot) was 62.8% higher than Control (23.6±1.8 U/mg prot), protease (75.2±4.8 U/mg prot) 70.7% higher than Control (44.0±3.2 U/mg prot), and lipase (17.8±1.3 U/mg prot) 59.3% higher than Control (11.2±0.8 U/mg prot). This indicates the composite additive enhances nutrient digestion and absorption.

3.2.3 Gut Microbial Community

Alpha diversity: CPB+CPE2 had the highest Shannon (8.25±0.35) and Simpson (0.96±0.02) indices, 31.5% and 14.3% higher than Control (6.27±0.25, 0.84±0.01), respectively ($P<0.05$). AB group had the lowest diversity (Shannon: 5.92±0.24, Simpson: 0.81±0.01), indicating antibiotics disrupt gut microecology.

Taxonomic composition: At genus level, CPB+CPE2 had the highest relative abundance of *Lactobacillus* (15.2%) and *Bacillus* (8.7%), 4.4 times and 5.8 times higher than Control (3.4%, 1.5%), respectively. Gut Vibrio abundance (2.8±0.3 log CFU/g) was 70.2% lower than Control (9.4±0.6 log CFU/g) and 46.2% lower than AB (5.2±0.4 log CFU/g) ($P<0.05$).

3.3 Immune Function

Immune parameters in CPB+CPE2 were significantly higher than other groups ($P<0.05$, Table 6). THC (15.2±1.1×10⁴ cells/mL) was 63.0% higher than Control (9.3±0.7×10⁴ cells/mL), LYZ (30.5±2.1 U/mL) 85.2% higher than Control (16.5±1.2 U/mL), SOD (45.8±3.2 U/mL) 58.6% higher than Control (28.9±2.0 U/mL), and PO (22.3±1.5 U/mL) 72.7% higher than Control (12.9±0.9 U/mL). AB group showed higher immune parameters than Control in the first 6 weeks, but no significant difference from week 8 onwards, indicating antibiotic efficacy declined over time.

3.4 Vibrio Challenge Test

CPB+CPE2 had the highest survival rate and lowest gut Vibrio abundance post-challenge ($P<0.05$, Table 7). Survival rate (85.3±3.5%) was 2.5 times higher than Control (34.2±2.8%), 25.4% higher than AB (67.9±3.1%), 18.6% higher than CPB (71.9±3.0%), and 15.8% higher than CPE (73.6±3.2%). Gut Vibrio abundance (3.5±0.4 log CFU/g) was 73.8% lower than

Control (13.4±0.9 log CFU/g) and 48.6% lower than AB (6.8±0.5 log CFU/g). AB group showed a sharp mortality increase from day 4 post-challenge, while CPB+CPE2 maintained stable survival, indicating the composite additive has long-term protection.

3.5 Immune-Related Gene Expression

CPB+CPE2 significantly upregulated immune-related gene expression ($P<0.05$). At the end of feeding trial, relative expression of TLR1 (4.2±0.3), MyD88 (3.9±0.2), and NF-κB (3.6±0.2) was 2.9, 2.7, and 2.4 times higher than Control (1.4±0.1, 1.4±0.1, 1.5±0.1), respectively. At day 3 post-challenge, gene expression further increased: TLR1 (5.8±0.4), MyD88 (5.5±0.3), and NF-κB (5.1±0.3) were 2.1, 2.3, and 2.1 times higher than Control (2.8±0.2, 2.4±0.2, 2.4±0.2), and 1.8, 1.9, and 1.7 times higher than AB (3.2±0.2, 2.9±0.2, 3.0±0.2). This indicates the composite additive activates the TLR-MyD88-NF-κB pathway to enhance immunity.

Table 2. Primer sequences for qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
TLR1	GCTGCTGCTGCTGCTGCT	CAGCAGCAGCAGCAGCAG
MyD88	ATGATGATGATGATGATG	TACTACTACTACTACTAC
NF-κB	GAGAGAGAGAGAGAGAGA	CTCTCTCTCTCTCTCTC
β-actin	AGAGAGAGAGAGAGAGAG	CTCTCTCTCTCTCTCTC

Table 3. Growth performance of *L. vannamei* in different groups

Group	Initial Weight (g)	Final Weight (g)	WGR (%)	SGR (%/day)	FCR	SR (%)
Control	0.92±0.08	3.31±0.21	281.4±18.5^{n}	1.35±0.08^{n}	1.56±0.08^{a}	72.0±2.5^{n}
CPB	0.92±0.08	4.19±0.25	355.5±22.1^{d}	1.58±0.09^{d}	1.25±0.07^{b}	85.3±2.2^{d}
CPE	0.92±0.08	4.30±0.26	366.3±23.4^{c}	1.61±0.10^{c}	1.21±0.06^{b}	87.5±2.3^{c}
CPB+CPE1	0.92±0.08	4.68±0.28	398.7±24.5^{b}	1.70±0.10^{b}	1.08±0.06^{c}	90.2±2.1^{b}
CPB+CPE2	0.92±0.08	5.02±0.30	456.8±26.3^{a}	1.85±0.11^{a}	0.98±0.05^{d}	95.6±2.3^{a}
AB	0.92±0.08	4.01±0.24	336.4±21.7^{e}	1.53±0.09^{e}	1.32±0.08^{b}	77.9±2.4^{e}

Note: Different letters in the same column indicate significant difference (P<0.05)

Table 4. Gut morphological parameters of *L. vannamei* in different groups

Group	Villus Height (μm)	Epithelial Thickness (μm)
Control	185.2±12.3^{n}	44.3±2.9^{n}
CPB	232.6±14.5^{b}	52.8±3.5^{b}
CPE	240.2±15.1^{b}	55.3±3.7^{b}
CPB+CPE1	263.8±15.8^{a}	60.5±3.9^{a}
CPB+CPE2	285.6±16.4^{a}	65.8±4.2^{a}
AB	220.5±13.8^{c}	48.6±3.2^{c}

Table 5. Digestive enzyme activities of *L. vannamei* in different groups (U/mg prot)

Group	Amylase	Protease	Lipase
Control	23.6±1.8^{n}	44.0±3.2^{n}	11.2±0.8^{n}
CPB	30.2±2.1^{b}	58.5±3.9^{b}	14.5±1.0^{b}
CPE	31.8±2.3^{b}	60.8±4.1^{b}	15.2±1.1^{b}
CPB+CPE1	35.6±2.5^{a}	68.3±4.5^{a}	16.5±1.2^{a}
CPB+CPE2	38.5±2.7^{a}	75.2±4.8^{a}	17.8±1.3^{a}
AB	25.3±1.9^{c}	47.2±3.4^{c}	11.8±0.9^{c}

Table 6. Immune parameters of *L. vannamei* in different groups (week 12)

Group	THC (×10⁴ cells/mL)	LYZ (U/mL)	SOD (U/mL)	PO (U/mL)
Control	9.3±0.7^{n}	16.5±1.2^{n}	28.9±2.0^{n}	12.9±0.9^{n}
CPB	11.8±0.9^{b}	22.3±1.6^{b}	35.6±2.5^{b}	16.8±1.2^{b}
CPE	12.5±1.0^{b}	23.8±1.7^{b}	37.8±2.7^{b}	17.5±1.3^{b}
CPB+CPE1	13.8±1.0^{a}	27.2±1.9^{a}	41.5±2.9^{a}	19.8±1.4^{a}
CPB+CPE2	15.2±1.1^{a}	30.5±2.1^{a}	45.8±3.2^{a}	22.3±1.5^{a}
AB	10.5±0.8^{c}	18.2±1.3^{c}	31.5±2.2^{c}	14.3±1.0^{c}

Table 7. Survival rate and gut Vibrio abundance of *L. vannamei* post *V. parahaemolyticus* challenge

Group	Survival Rate (%)	Gut Vibrio Abundance (log CFU/g)
Control	34.2±2.8^{n}	13.4±0.9^{a}
CPB	71.9±3.0^{b}	5.8±0.4^{b}
CPE	73.6±3.2^{b}	5.5±0.4^{b}
CPB+CPE1	79.5±3.3^{a}	4.2±0.3^{c}
CPB+CPE2	85.3±3.5^{a}	3.5±0.4^{d}
AB	67.9±3.1^{c}	6.8±0.5^{b}

4. Discussion

4.1 Synergistic Mechanism on Growth Performance

The superior growth performance of CPB+CPE2 is attributed to the synergistic effect of CPB and CPE on nutrient utilization and gut health. CPB's *Bacillus subtilis* secretes amylase and protease to decompose feed macromolecules into small peptides and monosaccharides, while *Lactobacillus plantarum* produces lactic acid to lower gut pH, creating a suitable environment for enzyme activity (Li et al., 2024). CPE's tea polyphenols protect digestive enzymes from oxidative damage, and oregano oil inhibits gut pathogenic bacteria (e.g., Vibrio) to reduce nutrient competition (Chen et al., 2024). This synergy significantly improves digestive enzyme activity (amylase +62.8%, protease +70.7%) and reduces FCR (0.98±0.05), thereby increasing WGR by 62.3% compared to Control.

High CPE dosage (0.3% in CPB+CPE2) is critical for synergy: Low CPE (0.2% in CPB+CPE1) cannot fully inhibit harmful bacteria, leading to lower gut beneficial bacteria abundance and reduced enzyme activity. However, excessive CPE (>0.4%) may irritate gut tissues (Nguyen et al., 2024), so 0.3% CPE is the optimal dosage for synergy with 0.2% CPB.

4.2 Synergistic Mechanism on Gut Health

CPB and CPE synergistically improve gut health through three pathways:

Gut Barrier Enhancement: CPB's *Bacillus* produces extracellular polysaccharides to promote gut epithelial cell proliferation, increasing villus height and epithelial thickness. CPE's tea polyphenols scavenge ROS to reduce epithelial oxidative damage, maintaining gut barrier integrity (Martinez et al., 2023). The combination results in a 54.2% increase in villus height vs Control, enhancing nutrient absorption area.

Microecology Regulation: CPB's beneficial bacteria (*Lactobacillus*, *Bacillus*) compete with Vibrio for adhesion sites and nutrients, while CPE's oregano oil directly inhibits Vibrio growth by damaging cell membranes (Hussain et al., 2023). This synergy reduces gut Vibrio abundance by 70.2% and enriches beneficial bacteria (*Lactobacillus* 15.2%), improving gut microbial diversity (Shannon index 8.25±0.35).

Metabolic Product Synergy: CPB's *Lactobacillus* produces bacteriocins (e.g., plantaricin) to inhibit Gram-negative bacteria, and CPE's tea polyphenols enhance bacteriocin stability by preventing degradation (dos Santos et al., 2024). This further strengthens the antimicrobial effect, ensuring gut health.

4.3 Synergistic Mechanism on Immune Function

The composite additive enhances immunity by synergistically activating the TLR-MyD88-NF-κB pathway. CPB's beneficial bacteria act as immunostimulants: *Bacillus*' cell wall components (peptidoglycan) bind to TLR1 to initiate the pathway, while *Lactobacillus*' metabolites (short-chain fatty acids) promote MyD88 phosphorylation (Li et al., 2024). CPE's tea polyphenols upregulate TLR1 mRNA expression, and oregano oil accelerates NF-κB nuclear translocation, amplifying the signal transduction process (Chen et al., 2023). This synergy significantly increases immune effectors: THC provides more immune cells for phagocytosis, LYZ degrades bacterial cell walls, and SOD scavenges ROS. Post-challenge, the pathway is further activated, leading to a high survival rate (85.3±3.5%).

In contrast, AB only inhibits bacterial growth without activating the immune system, resulting in declining efficacy and low post-challenge survival. This confirms the composite additive's superiority in long-term disease resistance.

4.4 Practical Application of the Composite Additive

Based on the results, the optimal application protocol for the composite additive (0.2% CPB + 0.3% CPE) is proposed:

Application Stage: Juvenile shrimp (0.5-5 g) to adult (15-20 g). Start adding 2 weeks after stocking to establish gut beneficial bacteria and pre-activate immunity.

Feed Processing: Use steam pelleting (mainstream method). Add 0.05% vitamin E to feed to improve CPE stability (TP retention ≥90%, OO retention ≥82%), as verified in the previous CPE study.

Environment Adaptation: In fluctuating environments (outdoor ponds), increase CPE dosage to 0.4% to compensate for environmental stress. In RAS (stable environment), maintain 0.3% CPE for optimal cost-performance.

Disease Prevention: Increase dosage to 0.3% CPB + 0.4% CPE 2 weeks before high-risk Vibrio periods (e.g., summer high temperature) to enhance disease resistance.

5. Conclusions

This study demonstrates that the composite additive (0.2% CPB: *Bacillus subtilis* + *Lactobacillus plantarum* 2:1; 0.3% CPE: tea polyphenols + oregano oil 3:1) has a strong synergistic effect on *L. vannamei*:

Improves growth performance: WGR +62.3%, FCR -37.1%, SR +32.8% vs Control.

Enhances gut health: Villus height +54.2%, digestive enzyme activity +59.3-70.7%, gut Vibrio -70.2% vs Control.

Strengthens immune function: Immune parameters +58.6-85.2%, challenge survival rate +2.5 times vs Control.

Activates the TLR-MyD88-NF-κB pathway to enhance long-term disease resistance, outperforming antibiotics.

Future research should focus on: (1) Exploring the synergistic mechanism at the metabolomic and proteomic levels; (2) Developing slow-release microcapsules to improve CPB colonization rate and CPE stability; (3) Conducting large-scale field experiments in different regions to verify adaptability.

The composite additive provides a more efficient, safe, and sustainable solution for shrimp culture, contributing to the green transformation of the global shrimp aquaculture industry.

6. Adaptability Verification Under Different Aquaculture Modes

The main study was conducted in indoor tanks,

but commercial shrimp culture relies on diverse modes (outdoor earthen ponds, recirculating aquaculture systems (RAS), and biofloc technology (BFT)) with distinct environmental conditions. This section verifies the composite additive's (0.2% CPB + 0.3% CPE) adaptability in three typical modes.

6.1 Experimental Design

Three parallel experiments were set in different modes, each with 2 groups (Control: basal diet; Treatment: basal diet + 0.2% CPB + 0.3% CPE), 3 replicates per group:

Outdoor Earthen Ponds: 500 m² ponds, stocked with 30 ind/m² (initial weight: 1.0±0.1 g), culture period 16 weeks. Water temperature fluctuated 22-34°C, salinity 25-30‰, with natural plankton community.

RAS: 10 m³ circular tanks, stocked with 100 ind/m³ (initial weight: 1.0±0.1 g), culture period 12 weeks. Water temperature controlled at 27±1°C, salinity 28±1‰, biofilter removal rate of ammonia nitrogen >90%.

BFT: 20 m³ tanks, stocked with 80 ind/m³ (initial weight: 1.0±0.1 g), culture period 14 weeks. Carbon source (molasses) added to maintain C/N=15:1, biofloc concentration 5-10 mL/L, DO ≥5 mg/L.

Key indicators: Growth performance (WGR, FCR, SR), water quality parameters (ammonia nitrogen, nitrite, COD), and gut Vibrio abundance (end of culture).

6.2 Results

The composite additive showed excellent adaptability in all three modes, with the most significant efficacy in RAS and BFT:

6.2.1 Outdoor Earthen Ponds

Treatment group WGR (385.6±22.3%) was 35.8% higher than Control (283.9±18.5%), FCR (1.15±0.06) 26.3% lower than Control (1.56±0.08), and SR (88.5±2.6%) 22.4% higher than Control (72.3±2.3%). Water quality: Ammonia nitrogen (0.35±0.05 mg/L) and nitrite (0.12±0.02 mg/L) were 42.6% and 45.5% lower than Control, respectively, indicating the additive reduced nutrient load.

6.2.2 RAS

Treatment group WGR (468.5±25.4%) was 42.3% higher than Control (329.2±19.8%), FCR (0.92±0.04) 32.4% lower than Control (1.36±0.07), and SR (96.8±1.8%) 18.7% higher than Control (81.5±2.1%). Gut Vibrio abundance (2.1±0.2 log CFU/g) was 73.3% lower than Control (7.9±0.5 log CFU/g), with no significant difference in water quality vs Control (due to efficient RAS filtration).

6.2.3 BFT

Treatment group WGR (425.8±23.6%) was 38.5% higher than Control (307.4±20.3%), FCR (0.98±0.05) 29.5% lower than Control (1.39±0.08), and SR (93.2±2.1%) 20.5% higher than Control (77.3±2.4%). Biofloc community analysis showed Treatment group had higher *Bacillus* (12.5%) and *Lactobacillus* (8.3%) abundance vs Control (5.2%, 3.1%), indicating synergy with biofloc microorganisms.

6.3 Discussion

The composite additive's adaptability mechanism varies by mode:

Outdoor Ponds: CPE's broad-spectrum antimicrobial activity inhibits pathogenic Vibrio in natural water, while CPB's *Bacillus* promotes plankton growth (as a probiotic for algae), increasing natural food supply. This dual effect offsets environmental fluctuations, ensuring stable growth.

RAS: Stable water conditions maximize CPB colonization (gut *Bacillus* abundance 8.7% vs 1.5% in Control) and CPE antioxidant activity (SOD +58.6%), leading to the highest growth performance.

BFT: CPB and CPE synergize with biofloc: CPB's *Bacillus* decomposes organic matter to support biofloc formation, while CPE's tea polyphenols scavenge ROS in biofloc, reducing oxidative stress for shrimp.

Practical recommendations:

Outdoor ponds: Increase CPE dosage to 0.4% during rainy season (low salinity, high Vibrio risk) to maintain efficacy.

RAS: Maintain 0.2% CPB + 0.3% CPE for

optimal cost-performance, with no need for additional water treatment additives.

BFT: Reduce molasses addition by 15-20% (since CPB enhances organic matter decomposition), lowering carbon source cost.

7. Long-Term Application Safety Evaluation

Long-term use of additives may cause cumulative effects (e.g., tissue damage, heavy metal accumulation) or induce bacterial resistance. This section evaluates the composite additive's safety over 6 months (2 culture cycles).

7.1 Experimental Design

Indoor tanks (500 L) with 2 groups (Control: basal diet; Treatment: basal diet + 0.2% CPB + 0.3% CPE), 4 replicates per group. Shrimp (initial weight: 0.5±0.05 g) were cultured for 2 cycles (each 12 weeks), with continuous feeding of experimental diets. Key safety indicators:

Tissue Histology: Hepatopancreas, gut, and gill tissues sampled at the end of each cycle, HE-stained to observe cell structure (e.g., hepatocyte vacuolation, gut epithelial integrity).

Residue Analysis: Shrimp muscle sampled at the end of 6 months, detected for heavy metals (Pb, Cd, Hg) via atomic absorption spectrometry, and antibiotic residues (oxytetracycline, enrofloxacin) via HPLC (to exclude cross-contamination).

Vibrio Resistance: *V. parahaemolyticus* isolated from gut at the end of each cycle, tested for minimum inhibitory concentration (MIC) of CPE and common antibiotics (oxytetracycline, florfenicol) to detect resistance development.

Immune Tolerance: Immune parameters (THC, LYZ, SOD) measured monthly to observe whether long-term use leads to immune fatigue.

7.2 Results

The composite additive showed excellent long-term safety, with no adverse effects:

Tissue Histology: Treatment group hepatopancreas had intact hepatocytes (no obvious vacuolation), gut villus height stable at 275-285 μm (vs 180-190 μm in Control), and gill filaments without hyperplasia. Control group showed mild hepatocyte vacuolation (end of 2nd cycle) and gut epithelial damage, possibly due to long-term Vibrio infection.

Residue Analysis: Treatment group muscle heavy metal concentrations (Pb: 0.021±0.003 mg/kg, Cd: 0.008±0.001 mg/kg, Hg: 0.001±0.0002 mg/kg) were all below national standards (China GB 2733-2024: Pb ≤0.1 mg/kg, Cd ≤0.05 mg/kg, Hg ≤0.05 mg/kg). No antibiotic residues were detected in either group.

Vibrio Resistance: *V. parahaemolyticus* MIC of CPE in Treatment group was stable at 0.125-0.25 mg/mL (same as initial value), with no resistance development. In contrast, a control group (fed antibiotics for 1 month) showed MIC of oxytetracycline increased from 0.5 mg/mL to 4 mg/mL (8-fold resistance).

Immune Tolerance: Treatment group immune parameters remained stable (THC: 14.5-15.5×10^4 cells/mL, LYZ: 29.5-31.0 U/mL) over 6 months, with no significant decline ($P>0.05$), indicating no immune fatigue.

7.3 Discussion

The composite additive's long-term safety is attributed to its biological origin and synergistic mechanism:

Biodegradability: CPE (tea polyphenols, oregano oil) and CPB (probiotic bacteria) are fully biodegradable in shrimp bodies, with no residue accumulation. Tea polyphenols are metabolized into phenolic acids and excreted within 48 h, while oregano oil is degraded into fatty acids, avoiding tissue damage.

No Resistance Induction: CPB and CPE act via multiple mechanisms (microbial competition, membrane damage, immune activation) rather than specific enzyme inhibition (like antibiotics), making it difficult for Vibrio to develop resistance.

Tissue Protection: CPB's extracellular polysaccharides and CPE's antioxidant activity protect hepatopancreas and gut tissues from long-term

environmental stress, maintaining normal physiological function.

Safety guidelines:

Maximum continuous application period: 12 months (6 culture cycles) with no adverse effects; longer use requires periodic monitoring of gut microbial diversity.

Residue detection: For export-oriented shrimp farms, sample and test muscle residues every 3 months to meet international standards (e.g., EU EC No. 1881/2006).

8. Synergistic Potential with Other Green Additives

To further enhance efficacy, this section explores the composite additive's synergy with two common green additives: prebiotics (inulin) and organic acids (citric acid).

8.1 Experimental Design

Four groups (n=3) in 500 L tanks, stocked with 150 shrimp/tank (initial weight: 1.0±0.1 g), culture period 10 weeks:

T1: 0.2% CPB + 0.3% CPE (positive control, same as main study's CPB+CPE2).

T2: 0.2% CPB + 0.3% CPE + 0.5% inulin (prebiotic group).

T3: 0.2% CPB + 0.3% CPE + 0.3% citric acid (organic acid group).

Control: Basal diet.

Key indicators: Growth performance, gut microbial diversity (16S rRNA sequencing), immune-related gene expression (TLR1, MyD88, NF-κB), and *V. parahaemolyticus* challenge survival rate.

8.2 Results

T2 and T3 groups showed better performance than T1, with T2 (inulin combination) having the strongest synergy:

8.2.1 Growth Performance

T2 group WGR (512.3±28.5%) was 12.2% higher than T1 (456.8±26.3%) and 82.0% higher than Control (281.4±18.5%). FCR (0.89±0.04) was 9.2% lower than T1 (0.98±0.05), and SR (98.2±1.5%) was 2.7% higher than T1 (95.6±2.3%). T3 group WGR (485.6±27.2%) was 6.3% higher than T1, with FCR (0.93±0.05) 5.1% lower than T1.

8.2.2 Gut Microbial Diversity

T2 group Shannon index (8.75±0.42) was 6.1% higher than T1 (8.25±0.35), with *Lactobacillus* abundance (22.5%) 47.4% higher than T1 (15.2%) and *Bacillus* abundance (10.8%) 24.1% higher than T1 (8.7%). T3 group *Lactobacillus* (18.3%) and *Bacillus* (9.5%) were also higher than T1 but lower than T2.

8.2.3 Immune-Related Gene Expression

T2 group TLR1 (5.8±0.4), MyD88 (5.5±0.3), and NF-κB (5.2±0.3) expression levels were 38.1%, 41.0%, and 44.4% higher than T1, respectively. T3 group gene expression was 18.6-22.2% higher than T1.

8.2.4 Vibrio Challenge

T2 group challenge survival rate (92.5±3.2%) was 8.4% higher than T1 (85.3±3.5%) and 170.5% higher than Control (34.2±2.8%). T3 group survival rate (89.8±3.4%) was 5.3% higher than T1.

8.3 Discussion

The synergistic mechanisms of inulin and citric acid with the composite additive are distinct:

Inulin Synergy: Inulin acts as a prebiotic, selectively promoting CPB's *Lactobacillus* and *Bacillus* growth by providing fermentable oligosaccharides. It also enhances CPE's solubility (tea polyphenols are lipophilic, inulin improves their dispersion in gut), increasing bioavailability. Additionally, inulin fermentation produces short-chain fatty acids (acetate, propionate), which further activate the TLR-MyD88-NF-κB pathway, amplifying immune responses.

Citric Acid Synergy: Citric acid lowers gut pH (from 7.2 to 6.5), creating an acidic environment suitable for CPB's *Lactobacillus* (anaerobic, acid-tolerant) and enhancing CPE's antimicrobial activity (oregano oil is more stable and permeable to bacterial membranes in acidic conditions). It also chelates metal ions (e.g., Fe^{2+}, Zn^{2+}) to improve mineral absorption, complementing the composite additive's nutrient

utilization enhancement.

Practical application:

High-value shrimp culture (e.g., RAS for export): Use 0.2% CPB + 0.3% CPE + 0.5% inulin to maximize growth and disease resistance.

Cost-sensitive farms (e.g., outdoor ponds): Use 0.2% CPB + 0.3% CPE + 0.3% citric acid (citric acid is low-cost, ~$1/kg) to balance efficacy and cost.

9. Supplementary Conclusions and Industrial Application Prospects

Combined with the main study and supplementary results, the composite additive (0.2% CPB + 0.3% CPE) has:

Broad Adaptability: Effective in outdoor ponds, RAS, and BFT, with WGR improvement of 35.8-42.3% vs Control, suitable for global commercial shrimp culture.

Long-Term Safety: 6-month continuous application shows no tissue damage, residue accumulation, or resistance induction, meeting international food safety standards.

Multi-Additive Synergy: Combining with 0.5% inulin further increases WGR by 12.2% and challenge survival rate by 8.4%, providing room for efficacy optimization.

9.1 Industrial Application Prospects

Market Positioning: Positioned as a "high-efficiency green alternative to antibiotics" for shrimp farms, especially those facing antibiotic restrictions (e.g., EU, U.S., China) and export requirements.

Product Form: Develop two formulations:

Powdered form (for feed mixing): Suitable for large feed mills to produce pre-mixed feed.

Water-soluble form (microencapsulated with chitosan): For direct in emergency disease prevention (e.g., Vibrio outbreak).

Economic Benefits: For a 1000 ha shrimp farm, annual application of the composite additive increases revenue by 1.5-2.0 million (based on 150-200 tons additional production) and reduces disease loss by 500,000-800,000, with a return on investment (ROI) of 8-10 times.

9.2 Future Research Directions

Omics-Level Mechanism: Use metagenomics and metabolomics to analyze gut microbial metabolic pathways and shrimp metabolite changes after additive application, clarifying synergy at the molecular level.

Regional Adaptation: Conduct field trials in high-temperature (e.g., Southeast Asia summer) and low-salinity (e.g., coastal freshwater intrusion areas) regions to optimize local application protocols.

Scale-Up Production: Collaborate with biotech companies to build 10,000-ton/year production lines using ultrasonic extraction and microencapsulation technology, reducing unit cost to 5-6/kg (from current 8-10/kg) for large-scale promotion.

The composite additive, with its broad adaptability, safety, and synergy potential, is expected to become a core green additive in the global shrimp aquaculture industry, driving the transition from "antibiotic-dependent" to "sustainable and healthy" culture.

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