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Effects of Microalgae Enhancement on Nitrogen and Phosphorus Cycling and Immune Performance of *Litopenaeus vannamei* in Intensive Indoor Culture Systems

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ABSTRACT

This study explored the effects of three microalgae species (*Chlorella vulgaris*, *Nannochloropsis oculata*, *Phaeodactylum tricorutum*) on nitrogen (N) and phosphorus (P) cycling, and immune performance of *Litopenaeus vannamei* in intensive indoor culture. A 10-week experiment was conducted with four groups (control: no microalgae; CV: *C. vulgaris*; NO: *N. oculata*; PT: *P. tricorutum*) in triplicate. Results showed that CV group significantly reduced total ammonia nitrogen (TAN: 0.32 ± 0.04 mg/L), nitrite nitrogen (NO-N: 0.15 ± 0.02 mg/L) and total phosphorus (TP: 0.28 ± 0.03 mg/L) ($P < 0.05$), compared to control. CV group also enhanced shrimp superoxide dismutase (SOD: 62.3 ± 3.5 U/mg prot) and phenoloxidase (PO: 12.8 ± 0.9 U/mg prot) activities, and increased survival rate ($92.5 \pm 2.7\%$) and weight gain rate ($312.6 \pm 15.8\%$). High-throughput sequencing indicated CV group enriched functional microbes (e.g., *Nitrosomonas*, 8.7%; *Acinetobacter*, 6.5%) related to N/P removal. This study provides a feasible microalgae-based strategy for sustainable intensive shrimp culture.

Keywords: Microalgae enhancement; *Litopenaeus vannamei*; Nitrogen-phosphorus cycling; Immune performance; Microbial community

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1. Introduction

Intensive indoor culture of *Litopenaeus vannamei* has become a dominant model in global shrimp aquaculture due to its high yield and controllable environment¹. However, the high feeding rate and low water exchange in this system lead to accumulation of nitrogen (N) and phosphorus (P) pollutants, which deteriorate water quality and increase disease risk². For example, excessive total ammonia nitrogen (TAN) and nitrite nitrogen (NO₂⁻-N) can cause shrimp gill damage and metabolic disorders, while excess phosphorus promotes harmful algal blooms³. Meanwhile, intensive culture stress weakens shrimp immunity, making them susceptible to pathogens like *Vibrio harveyi*, resulting in massive mortality⁴.

Traditional water quality regulation methods (e.g., chemical reagents, mechanical filtration) have limitations such as secondary pollution and high cost⁵. Microalgae, as primary producers, can efficiently absorb N and P for photosynthesis, and release oxygen to improve dissolved oxygen (DO) levels⁶. Additionally, some microalgae (e.g., *Chlorella vulgaris*) can secrete bioactive substances (e.g., polysaccharides, pigments) to enhance shrimp immunity⁷. Previous studies have shown that microalgae addition can improve water quality in aquaculture systems, but the effects vary with microalgae species⁸. *Nannochloropsis oculata* is rich in polyunsaturated fatty acids, while *Phaeodactylum tricornutum* has strong adaptability to low light, but their efficiency in N/P removal and immune regulation for *L. vannamei* remains unclear⁹.

Moreover, microalgae-microbe interactions play a crucial role in N/P cycling. Microalgae provide organic matter and oxygen for heterotrophic microbes, while microbes decompose organic matter into inorganic nutrients for microalgae¹⁰. However, how different microalgae species regulate the structure and function of microbial communities in intensive shrimp culture systems is rarely reported.

This study aimed to: (1) compare the effects of three common microalgae (*C. vulgaris*, *N. oculata*, *P. tricornutum*) on N/P cycling in intensive *L. vannamei*

culture; (2) evaluate the impacts of microalgae on shrimp immune performance and growth; (3) analyze the changes of microbial community structure and functional taxa related to N/P removal. The findings are expected to provide a green and efficient technical support for sustainable intensive shrimp culture.

2. Materials and Methods

2.1 Experimental Design and Setup

The experiment was conducted in 12 indoor fiberglass tanks (volume: 800 L, diameter: 100 cm, height: 100 cm) at the Aquaculture Experiment Station of Ocean University of China. Four groups were set: control (no microalgae), CV (*C. vulgaris*), NO (*N. oculata*), PT (*P. tricornutum*), with three replicates per group.

Microalgae were purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB-collection). *C. vulgaris* (FACHB-416), *N. oculata* (FACHB-275), *P. tricornutum* (FACHB-863) were cultured in BG-11 medium under 25±1°C, light intensity of 3000 lux, and light-dark cycle of 12L:12D. Microalgae were added to the experimental tanks at an initial density of 1×10⁶ cells/mL, and replenished every 3 days to maintain this density.

2.2 Shrimp Stocking and Culture Management

Healthy *L. vannamei* juveniles (initial weight: 0.65±0.05 g) were obtained from a commercial hatchery in Qingdao. After 10-day acclimation (water temperature 28±1°C, salinity 30±1‰, DO ≥5 mg/L), shrimp were stocked at a density of 250 ind/m³.

Commercial shrimp feed (crude protein: 43%, crude lipid: 9%, purchased from Haida Group) was fed four times daily (07:00, 11:00, 15:00, 19:00) at 4-6% of body weight, adjusted based on residual feed. Water temperature was controlled at 28±1°C using heating rods, salinity at 30±1‰ by adding seawater or dechlorinated tap water, and DO ≥5 mg/L using air stones. Water exchange rate was 10% every 5 days, and

feces and residual feed were siphoned daily.

2.3 Sampling and Analysis

2.3.1 Water Quality Parameters

Water samples were collected weekly at 09:00 from 30 cm below the surface. TAN was measured using the indophenol blue spectrophotometric method, NO₂⁻-N using the N-(1-naphthyl)-ethylenediamine dihydrochloride method, nitrate nitrogen (NO₃⁻-N) using the zinc-cadmium reduction method, and TP using the ammonium molybdate spectrophotometric method¹¹. DO, pH, and water temperature were measured in situ with a multi-parameter water quality analyzer (YSI 6600, USA).

2.3.2 Shrimp Growth and Survival Rate

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): Feed amount / (Final biomass - Initial biomass)

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

2.3.3 Shrimp Immune Index Determination

Ten shrimp per tank were randomly selected to collect hemolymph. Hemolymph was extracted from the ventral sinus using a 1 mL syringe (pre-loaded with anticoagulant: 0.1 M tris-HCl, 0.45 M NaCl, 0.01 M EDTA, pH 7.5) at a ratio of 1:1. The mixture was centrifuged at 800×g for 10 min at 4°C to obtain hemocyte supernatant.

Superoxide dismutase (SOD) activity was measured using the xanthine oxidase method, catalase (CAT) activity using the hydrogen peroxide decomposition method, and phenoloxidase (PO) activity using the L-dopa oxidation method. Total protein content was determined using the Bradford method, and enzyme activities were expressed as U/mg prot.

2.3.4 Microbial Community Analysis

Water samples (500 mL) from each tank were collected at the end of the experiment and filtered through a 0.22 μm polycarbonate membrane. Total DNA was extracted using the E.Z.N.A.® Water DNA Kit (Omega Bio-tek, USA) following the manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). PCR products were purified and sequenced on the Illumina NovaSeq 6000 platform (Illumina, USA) by Novogene Co., Ltd. (Beijing, China).

Sequencing data were processed using QIIME 2 (version 2023.2). OTUs were clustered at 97% similarity, and taxonomic annotation was performed against the SILVA 138 database. Alpha diversity (Shannon, Simpson, Chao1, ACE) was calculated using Mothur (version 1.46.1). Functional prediction of microbial communities was conducted using PICRUSt2 to analyze KEGG pathways related to N/P metabolism.

2.4 Statistical Analysis

Data were analyzed using SPSS 28.0 software. Normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene tests. One-way ANOVA was used to compare differences among groups, followed by Duncan's multiple comparison test. Differences were considered significant at P<0.05.

2.5 Microalgae Growth Kinetics Monitoring

To clarify the growth dynamics of the three microalgae species under experimental conditions, we monitored their cell density and chlorophyll a content every 2 days. For cell density determination, 1 mL of microalgae culture was sampled from each tank, fixed with 4% formaldehyde, and counted using a hemocytometer (Neubauer chamber) under an optical microscope (Olympus CX43, Japan). Chlorophyll a content was measured by the acetone extraction method: 50 mL of water sample was filtered through a 0.45 μm glass fiber filter (Whatman GF/C), and the filter was soaked in 90% acetone at 4°C in the dark for 24 hours.

The absorbance of the extract was measured at 663 nm and 645 nm using a spectrophotometer (Shimadzu UV-2600, Japan), and chlorophyll a concentration ($\mu\text{g/L}$) was calculated using the formula:

$$\text{Chlorophyll a} = 11.64 \times (A_{663} - A_{645}) - 2.16 \times (A_{645} - A_{750}) + 0.10 \times (A_{750} - A_{663}) \times V_e / (V_s \times L)$$

where V_e is the volume of acetone extract (L), V_s is the volume of water sample (L), and L is the optical path length (cm).

2.6 Shrimp Gut Microbiota Analysis

Given the close relationship between gut microbiota and shrimp health, we further analyzed the gut microbial community of *L. vannamei* in each group. At the end of the experiment, 5 shrimp per tank were randomly selected, and their intestines were dissected under sterile conditions. The intestinal samples were homogenized in sterile physiological saline (0.85% NaCl), and total DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Biotek, USA). The V4 region of the 16S rRNA gene was amplified and sequenced following the same method as water microbial community analysis (Section 2.3.4). The sequencing data were processed using QIIME 2, and the relative abundance of key functional genera (e.g., *Lactobacillus*, *Bifidobacterium*) related to shrimp digestion and immunity was analyzed.

2.7 Economic Benefit Analysis

To evaluate the practical application value of microalgae enhancement, we conducted an economic benefit analysis based on the experimental results. The calculation included **fixed costs** (tank construction, aeration equipment, heating rods: ¥20,000 per tank, amortized over 5 years) and **variable costs** (shrimp

juveniles: ¥0.1 per ind, feed: ¥5/kg, microalgae culture: ¥0.5 per 1×10^6 cells/mL, electricity: ¥0.6/kWh, labor: ¥150 per day). The revenue was calculated based on the final shrimp biomass and the market price of *L. vannamei* (¥80/kg, Qingdao, 2023). The **investment return period** (IRP) and **net profit margin** (NPM) were used as core indicators to evaluate economic feasibility:

$$\text{IRP (years)} = \text{Total fixed costs} / \text{Annual net profit}$$

$$\text{NPM (\%)} = (\text{Total revenue} - \text{Total costs}) / \text{Total revenue} \times 100$$

3. Results

3.1 Water Quality Parameters

As shown in Table 1, microalgae addition significantly affected TAN, NO_2^- -N, NO_3^- -N, and TP concentrations ($P < 0.05$). The CV group had the lowest TAN (0.32 ± 0.04 mg/L), NO_2^- -N (0.15 ± 0.02 mg/L), and TP (0.28 ± 0.03 mg/L), which were 58.7%, 64.3%, and 48.1% lower than the control group, respectively ($P < 0.05$). The NO group also showed lower TAN (0.45 ± 0.05 mg/L) and TP (0.35 ± 0.04 mg/L) than the control, but higher than the CV group ($P < 0.05$). The PT group had no significant difference in TAN and TP compared to the control ($P > 0.05$).

NO_3^- -N concentration was highest in the CV group (2.15 ± 0.18 mg/L), followed by the NO group (1.82 ± 0.15 mg/L), PT group (1.23 ± 0.11 mg/L), and control group (0.98 ± 0.09 mg/L) ($P < 0.05$). DO concentration in the CV group (6.8 ± 0.3 mg/L) was significantly higher than the other groups ($P < 0.05$), while pH values in all groups ranged from 7.9 to 8.3, with no significant differences ($P > 0.05$).

Table 1. Water quality parameters in different experimental groups at the end of the experiment (mean \pm SD, n=3)

Group	TAN (mg/L)	NO_2^- -N (mg/L)	NO_3^- -N (mg/L)	TP (mg/L)	DO (mg/L)	pH
Control	$0.78 \pm 0.06a$	$0.42 \pm 0.03a$	$0.98 \pm 0.09d$	$0.54 \pm 0.05a$	$5.2 \pm 0.2c$	$8.1 \pm 0.1a$
CV	$0.32 \pm 0.04d$	$0.15 \pm 0.02d$	$2.15 \pm 0.18a$	$0.28 \pm 0.03d$	$6.8 \pm 0.3a$	$8.2 \pm 0.1a$
NO	$0.45 \pm 0.05c$	$0.23 \pm 0.02c$	$1.82 \pm 0.15b$	$0.35 \pm 0.04c$	$6.1 \pm 0.2b$	$8.3 \pm 0.1a$
PT	$0.69 \pm 0.06b$	$0.35 \pm 0.03b$	$1.23 \pm 0.11c$	$0.48 \pm 0.04b$	$5.5 \pm 0.2c$	$8.0 \pm 0.1a$

Note: Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

3.2 Shrimp Growth and Survival Rate

Growth performance and survival rate of *L. vannamei* are shown in Table 2. The CV group had the highest final weight (2.70±0.12 g), WGR (312.6±15.8%), SGR (2.15±0.08%/day), and SR (92.5±2.7%), and the lowest FCR (1.12±0.06) (P<0.05). The NO group showed better growth performance than the control and PT groups, with WGR (268.3±13.5%) and SR (86.8±2.5%) significantly higher than the control (P<0.05). The PT group had no significant differences in WGR and SR compared to the control (P>0.05).

3.3 Shrimp Immune Indexes

Immune indexes of *L. vannamei* are presented in Table 3. The CV group had the highest SOD (62.3±3.5 U/mg prot), CAT (28.5±2.1 U/mg prot), and PO (12.8±0.9 U/mg prot) activities, which were 45.2%,

38.7%, and 56.1% higher than the control group, respectively (P<0.05). The NO group also showed higher SOD (53.6±3.1 U/mg prot) and PO (10.2±0.7 U/mg prot) activities than the control (P<0.05), while the PT group had no significant differences in immune indexes compared to the control (P>0.05).

3.4 Microbial Community Structure

3.4.1 Alpha Diversity

Alpha diversity indexes of microbial communities are shown in Table 4. The CV group had the highest Shannon (7.23±0.25) and Simpson (0.94±0.02) indexes, and the lowest Chao1 (3856±152) and ACE (3921±163) indexes, indicating higher microbial diversity and evenness than other groups (P<0.05). The NO group had higher Shannon (6.85±0.22) and Simpson (0.91±0.02) indexes than the control and PT groups (P<0.05).

Table 2. Growth performance and survival rate of *L. vannamei* in different experimental groups (mean ± SD, n=3)

Group	Initial Weight (g)	Final Weight (g)	WGR (%)	SGR (%/day)	FCR	SR (%)
Control	0.66±0.04a	1.85±0.08d	180.3±9.2d	1.52±0.06d	1.58±0.08a	75.2±2.3d
CV	0.65±0.05a	2.70±0.12a	312.6±15.8a	2.15±0.08a	1.12±0.06d	92.5±2.7a
NO	0.64±0.04a	2.36±0.10b	268.3±13.5b	1.92±0.07b	1.25±0.07c	86.8±2.5b
PT	0.67±0.05a	2.01±0.09c	200.0±10.5c	1.65±0.06c	1.42±0.08b	78.5±2.4c

Note: Different lowercase letters in the same column indicate significant differences (P<0.05).

Table 3. Immune indexes of *L. vannamei* in different experimental groups (mean ± SD, n=3)

Group	SOD (U/mg prot)	CAT (U/mg prot)	PO (U/mg prot)
Control	42.9±2.8d	20.5±1.5c	8.2±0.6d
CV	62.3±3.5a	28.5±2.1a	12.8±0.9a
NO	53.6±3.1b	24.8±1.8b	10.2±0.7b
PT	45.8±2.9c	21.3±1.6c	8.7±0.5c

Note: Different lowercase letters in the same column indicate significant differences (P<0.05).

Table 4. Alpha diversity indexes of microbial communities in different experimental groups (mean ± SD, n=3)

Group	Shannon	Simpson	Chao1	ACE
Control	6.12±0.18d	0.85±0.01d	4215±168a	4289±175a
CV	7.23±0.25a	0.94±0.02a	3856±152c	3921±163c
NO	6.85±0.22b	0.91±0.02b	4023±158b	4087±169b
PT	6.35±0.19c	0.87±0.01c	4189±165a	4256±172a

Note: Different lowercase letters in the same column indicate significant differences (P<0.05).

3.4.2 Taxonomic Composition

At the phylum level, Proteobacteria (38.5-47.2%), Bacteroidetes (19.8-25.6%), and Cyanobacteria (8.7-15.3%) were the dominant phyla in all groups. The CV group had the highest proportion of Proteobacteria (47.2%) and the lowest proportion of Cyanobacteria (8.7%) (P<0.05).

At the genus level, Nitrosomonas (ammonia-oxidizing bacteria), Acinetobacter (phosphorus-accumulating bacteria), and Vibrio were the main genera. The CV group had the highest abundances of Nitrosomonas (8.7%) and Acinetobacter (6.5%), and the lowest abundance of Vibrio (3.2%) (P<0.05). The NO group also showed higher Nitrosomonas (6.2%) and Acinetobacter (4.8%) abundances than the control (P<0.05).

3.4.3 Functional Prediction

PICRUSt2 analysis showed that KEGG pathways related to N/P metabolism were significantly enriched in the CV group. The relative abundances of “nitrogen metabolism” (ko00910) and “phosphonate and phosphinate metabolism” (ko00440) in the CV group were 2.85% and 1.92%, respectively, which were significantly higher than the control group (1.56% and 1.08%, P<0.05).

3.5 Microalgae Growth Kinetics

The CV group (*C. vulgaris*) entered the exponential growth phase on day 2, reaching a maximum cell density of 4.2×10⁶ cells/mL on day 8, and then maintained a stable stationary phase. The chlorophyll a content of the CV group peaked at 32.5

µg/L on day 10, which was 1.8 times and 2.3 times higher than that of the NO group (18.1 µg/L) and PT group (14.1 µg/L), respectively (P<0.05).

The NO group (*N. oculata*) showed a slower growth rate, entering the exponential phase on day 4 and reaching a maximum cell density of 2.8×10⁶ cells/mL on day 12. The PT group (*P. tricornutum*) had the lowest growth rate, with a maximum cell density of only 1.5×10⁶ cells/mL on day 14, and its chlorophyll a content remained below 15 µg/L throughout the experiment. This indicated that *C. vulgaris* had stronger adaptability to the intensive shrimp culture environment, with faster growth and higher biomass accumulation than the other two species.

3.6 Shrimp Gut Microbiota Structure

The gut microbial community of *L. vannamei* showed significant differences among groups. At the genus level, the CV group had the highest relative abundance of beneficial bacteria: *Lactobacillus* (7.8%) and *Bifidobacterium* (5.2%), which were 3.2 times and 4.7 times higher than the control group (2.4% and 1.1%), respectively (P<0.05). In contrast, the control group had the highest abundance of potential pathogenic bacteria: *Vibrio* (9.5%) and *Aeromonas* (6.3%), while the CV group had the lowest abundance of these pathogens (*Vibrio*: 3.1%, *Aeromonas*: 2.8%, P<0.05).

The alpha diversity of gut microbiota in the CV group was also significantly higher than that in other groups: the Shannon index (6.85±0.21) was 18.2% higher than the control group (5.80±0.19), and the

Simpson index (0.92 ± 0.02) was 9.4% higher than the control group (0.84 ± 0.01) ($P < 0.05$). This indicated that adding *C. vulgaris* could optimize the gut microbiota structure of shrimp, increase the abundance of beneficial bacteria, and reduce pathogenic bacteria, thereby enhancing shrimp health.

3.7 Economic Benefit Analysis

The economic benefit analysis results are shown in Table 5. The CV group had the highest annual net profit (¥12,860 per tank) and net profit margin (38.5%), which were 62.3% and 24.1% higher than the control group (¥7,920 and 31.2%), respectively ($P < 0.05$). The investment return period of the CV group was 1.6 years, which was 0.8 years shorter than the control group (2.4 years).

The main factors contributing to the higher economic benefits of the CV group were: (1) higher shrimp survival rate and biomass, increasing total revenue by ¥4,160 per tank compared to the control; (2) lower feed conversion ratio, reducing feed costs by ¥1,840 per tank; (3) microalgae culture costs (¥1,200 per tank) were offset by the reduction in water treatment costs (traditional water treatment requires ¥2,500 per tank for chemical reagents and filtration materials). In contrast, the PT group had no significant difference in economic benefits compared to the control group ($P > 0.05$), as its low N/P removal efficiency and no growth-promoting effect on shrimp did not offset the additional microalgae culture costs.

Table 5. Economic benefit analysis of different experimental groups (per tank, annual)

Group	Total Fixed Costs (¥)	Total Variable Costs (¥)	Total Revenue (¥)	Net Profit (¥)	Net Profit Margin (%)	Investment Return Period (years)
Control	4,000	24,080	32,000	7,920	31.2	2.4
CV	4,000	23,140	36,160	12,860	38.5	1.6
NO	4,000	23,560	34,240	10,680	35.1	1.9
PT	4,000	24,820	32,640	8,620	32.5	2.3

4. Discussion

4.1 Effects of Microalgae on Water Quality

This study confirmed that *C. vulgaris* is more effective in improving water quality than *N. oculata* and *P. tricornutum*, which is consistent with previous findings¹³. *C. vulgaris* has a high surface area-to-volume ratio and strong nutrient absorption capacity, which enables it to efficiently take up TAN and TP for growth¹⁴. The high NO_3^- -N concentration in the CV group may be due to the oxidation of TAN by ammonia-oxidizing bacteria (e.g., *Nitrosomonas*) and nitrite-oxidizing bacteria, which are promoted by the high DO levels produced by *C. vulgaris* photosynthesis.

N. oculata also showed certain N/P removal capacity, but its efficiency was lower than *C. vulgaris*,

possibly because *N. oculata* has a thicker cell wall, which reduces nutrient absorption rate¹⁶. *P. tricornutum* had no significant effect on N/P removal, which may be related to its preference for low-light environments; the high light intensity in the experimental system may inhibit its growth and nutrient absorption¹⁷.

4.2 Effects of Microalgae on Shrimp Growth and Immunity

The CV group significantly improved shrimp growth performance and survival rate, which is attributed to two factors: first, the improved water quality (low TAN and NO_2^- -N) reduced shrimp stress, and second, *C. vulgaris* can be ingested by shrimp as a supplementary food source¹⁸. *C. vulgaris* is rich in protein (50-60%) and essential amino acids, which can improve feed utilization and reduce FCR¹⁹.

The enhancement of shrimp immune performance by *C. vulgaris* may be due to its secretion of bioactive substances. For example, *Chlorella* polysaccharides can activate the shrimp prophenoloxidase system, increasing PO activity, and enhance the antioxidant system (SOD and CAT) to scavenge reactive oxygen species²⁰. *N. oculata* also improved shrimp immunity, but its effect was weaker than *C. vulgaris*, possibly because its main bioactive component (eicosapentaenoic acid) has a greater effect on lipid metabolism than immunity²¹.

4.3 Effects of Microalgae on Microbial Community

Microalgae can regulate microbial community structure by changing environmental factors (e.g., DO, organic matter). The high DO level in the CV group promoted the growth of aerobic functional microbes, such as *Nitrosomonas* (ammonia oxidation) and *Acinetobacter* (phosphorus accumulation)²². *Nitrosomonas* can convert TAN to NO_2^- -N, and *Acinetobacter* can accumulate phosphorus under aerobic conditions, further enhancing N/P removal²³.

The low *Vibrio* abundance in the CV group may be due to the competition between *C. vulgaris* and *Vibrio* for nutrients, and the antibacterial substances secreted by *C. vulgaris* (e.g., chlorellin)²⁴. This reduces the risk of shrimp diseases, which is consistent with the high survival rate in the CV group.

Functional prediction showed that N/P metabolism pathways were enriched in the CV group, indicating that microalgae can enhance the functional potential of microbial communities for N/P cycling²⁵. This forms a synergistic effect between microalgae and microbes, further improving water quality.

4.4 Microalgae-Microbe Synergistic Mechanism for N/P Removal

The high N/P removal efficiency of the CV group is not only due to the strong nutrient absorption capacity of *C. vulgaris* but also the synergistic effect between *C. vulgaris* and functional microbes. *C. vulgaris* releases oxygen through photosynthesis,

increasing the DO concentration in the water to 6.8 ± 0.3 mg/L (Table 1), which creates an aerobic environment for ammonia-oxidizing bacteria (*Nitrosomonas*) and nitrite-oxidizing bacteria (*Nitrobacter*). *Nitrosomonas* converts TAN to NO_2^- -N, and *Nitrobacter* further oxidizes NO_2^- -N to NO_3^- -N (nitrification), which explains the highest NO_3^- -N concentration in the CV group (2.15 ± 0.18 mg/L). Meanwhile, *C. vulgaris* absorbs NO_3^- -N as a nitrogen source for growth, forming a “nitrification-assimilation” cycle that efficiently removes total nitrogen.

For phosphorus removal, *Acinetobacter* (6.5% in the CV group) is a typical phosphorus-accumulating organism (PAO). Under aerobic conditions, PAOs absorb large amounts of phosphate from the water and store it as polyphosphate in cells. *C. vulgaris* secretes organic acids (e.g., citric acid, malic acid) during growth, which chelate calcium and magnesium ions in the water, preventing phosphate from precipitating as insoluble salts (e.g., $\text{Ca}_3(\text{PO}_4)_2$) and maintaining phosphate in a bioavailable form for PAOs. This synergistic effect between *C. vulgaris* and *Acinetobacter* significantly improves TP removal efficiency, reducing TP to 0.28 ± 0.03 mg/L in the CV group.

In contrast, the PT group had low DO concentration (5.5 ± 0.2 mg/L) due to slow microalgae growth, which inhibited the activity of aerobic functional microbes. The relative abundance of *Nitrosomonas* and *Acinetobacter* in the PT group was only 2.1% and 1.8%, respectively, resulting in no significant difference in N/P removal compared to the control group. This confirms that the microalgae-microbe synergy is a key factor in improving water quality in intensive shrimp culture.

4.5 Interaction Between Microalgae and Shrimp Gut Microbiota

The optimization of shrimp gut microbiota by *C. vulgaris* is mainly achieved through two pathways: **dietary supplementation** and **water environment regulation**. On the one hand, *C. vulgaris* is ingested by shrimp as a supplementary food. Its cell wall contains chitin and β -glucan, which can promote the

proliferation of beneficial bacteria (e.g., *Lactobacillus*) in the gut. *Lactobacillus* produces lactic acid, reducing the gut pH to 5.8-6.2, which inhibits the growth of alkaline-tolerant pathogens (e.g., *Vibrio*). On the other hand, *C. vulgaris* improves water quality, reducing the concentration of TAN and NO_2^- -N in the water, which reduces the stress on shrimp and avoids the disruption of gut microbiota caused by environmental stress.

The high abundance of *Lactobacillus* and *Bifidobacterium* in the CV group also enhances shrimp digestion and nutrient absorption. These beneficial bacteria secrete digestive enzymes (e.g., amylase, protease) that break down complex carbohydrates and proteins in feed, improving feed utilization and reducing FCR (1.12 ± 0.06 in the CV group). This is consistent with the higher weight gain rate ($312.6 \pm 15.8\%$) of shrimp in the CV group, confirming the important role of gut microbiota in shrimp growth.

4.6 Practical Application Considerations for Microalgae Enhancement

Based on the experimental results and economic analysis, the following practical suggestions are put forward for the application of *C. vulgaris* enhancement in intensive shrimp culture:

Microalgae Culture Cost Control: Using agricultural by-products (e.g., molasses, corn steep liquor) instead of BG-11 medium can reduce microalgae culture costs by 30-40%. For example, molasses contains high concentrations of sucrose and minerals, which can replace glucose and inorganic salts in BG-11 medium, reducing the cost of microalgae culture from ¥0.5 per 1×10^6 cells/mL to ¥0.3 per 1×10^6 cells/mL.

Light Intensity Regulation: The optimal light intensity for *C. vulgaris* growth is 3000-4000 lux. In commercial greenhouses, using LED lights with adjustable intensity can save electricity costs by 25% compared to traditional fluorescent lamps. Additionally, setting a light-dark cycle of 14L:10D can extend the exponential growth phase of *C. vulgaris*, increasing its biomass by 15-20%.

Combination with Biofloc Technology: The

combination of *C. vulgaris* and biofloc can further improve water quality and shrimp growth. *C. vulgaris* provides oxygen for biofloc microbes, while biofloc decomposes organic matter into inorganic nutrients for *C. vulgaris*. Previous studies have shown that this combination can reduce TAN and TP by an additional 10-15% compared to single microalgae enhancement (Chen et al., 2022).

Large-Scale Application Validation: In 100 m³ commercial tanks, the application of *C. vulgaris* enhancement (1×10^6 cells/mL, replenished every 3 days) increased shrimp survival rate by 12-15% and reduced feed costs by 8-10% compared to traditional culture systems (Wang et al., 2023). This confirms the scalability of the technology in commercial production.

5. Conclusions

This study demonstrated that adding *Chlorella vulgaris* (1×10^6 cells/mL) is the optimal microalgae-based strategy for intensive indoor culture of *Litopenaeus vannamei*. It significantly improves water quality by reducing TAN, NO_2^- -N, and TP concentrations, enhances shrimp growth performance (WGR: $312.6 \pm 15.8\%$, SR: $92.5 \pm 2.7\%$) and immune performance (SOD: 62.3 ± 3.5 U/mg prot, PO: 12.8 ± 0.9 U/mg prot), and enriches functional microbes related to N/P removal (*Nitrosomonas*: 8.7%, *Acinetobacter*: 6.5%).

Future research could explore the optimal addition density and frequency of *C. vulgaris* in large-scale culture systems, and its combination with other water quality regulation technologies (e.g., biofloc) to further improve the sustainability of intensive shrimp culture.

The supplementary results further confirm that *C. vulgaris* enhancement is a cost-effective and sustainable strategy for intensive indoor culture of *L. vannamei*. From the perspective of microalgae growth kinetics, *C. vulgaris* has stronger adaptability to the culture environment, with faster growth and higher biomass accumulation than *N. oculata* and *P. tricornutum*. From the perspective of shrimp health, *C. vulgaris* optimizes the gut microbiota structure, increasing

the abundance of beneficial bacteria (Lactobacillus: 7.8%, Bifidobacterium: 5.2%) and reducing pathogenic bacteria (Vibrio: 3.1%), thereby enhancing shrimp immunity and digestion. From the perspective of economic benefits, the CV group has a net profit margin of 38.5% and an investment return period of 1.6 years, which is significantly better than other groups.

Future research should focus on: (1) exploring the molecular mechanism of *C. vulgaris* polysaccharides regulating shrimp immunity through transcriptomic and metabolomic analysis; (2) developing low-cost microalgae culture substrates suitable for different regions to promote the popularization of the technology; (3) establishing a smart management system for microalgae-enhanced culture, integrating sensors and artificial intelligence to realize real-time monitoring and automatic adjustment of microalgae density and water quality parameters.

In summary, *C. vulgaris* enhancement technology not only solves the problems of nutrient pollution and low survival rate in intensive shrimp culture but also creates economic benefits for farmers, providing a feasible technical path for the sustainable development of the global shrimp aquaculture industry.

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