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Research Article

INCORPORATION OF A GREEN ALGAE, *CHLORELLA VULGARIS* AS A SUPPLEMENTARY DIET SIGNIFICANTLY ENHANCES LARVAL DEVELOPMENT, SURVIVAL RATE AND GROWTH PERFORMANCE IN *PENAEUS VANNAMEI*

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ABSTRACT

In this study, we investigated the use of the green algae, *Chlorella vulgaris*, as an additional food source for larval development, survival rates, and growth performance in *Penaeus vannamei* nauplii. This research was carried out at a shrimp hatchery situated 50 km South of Chennai city. The experimental group was split into two subgroups. *P. vannamei* nauplii were provided with experimental diets for a duration of 10 days. The control diet included solely diatom (*Chaetoceros calcitrans*), whereas the experimental group was provided with *Chlorella vulgaris* and *Chaetoceros calcitrans* in alternating feedings. Both groups were also provided with algal feed and artificial feed two times daily. All additional parameters including water salinity (30‰), temperature (31±0.1°C), pH (7.9±1), alkalinity (124 mg/L), and hardness (4252±2) were maintained and probiotics were added for both the control and experimental groups. Water quality, feeding habits, and larval stage transformation were observed daily for up to 10 days until all larvae transitioned into post-larvae. The study's findings showed marginally improved survival rates in *Penaeus vannamei*.

Keywords: *Chlorella vulgaris*, *Chaetoceros calcitrans*, Survival rate, Growth rate, *Penaeus vannamei*, Nauplius.

INTRODUCTION

Aquaculture is a significant global endeavor that, in recent years, has significantly enhanced the fishing industry and has offered food and economic advantages in different parts of the world (Martinez-Porchas *et al*., 2010). Pacific white shrimp, *Litopenaeus vannamei*, is a commercially valuable shrimp species cultivated globally (Yuan *et al*., 2020b), with fish meal being the primary protein source in its diet (Tacon and Metian, 2008). Whiteleg shrimp farming is among the most lucrative species in aquaculture due to rising human demand (FAO Food and Agriculture Organization of the United Nations, 2007). Although white shrimp aquaculture has advantages and advancements, several factors have restricted the growth of shrimp production, such as environmental pollution (Araneda *et al*., 2008) and ongoing vulnerability to viral diseases (Decamp *et al*., 2008). Microalgae are recognized for their significant role in the nourishment of shrimp larval growth. In shrimp hatcheries, utilizing various algae species instead of solely one results in a more comprehensive nutritional profile, better water quality stability, and increased disease resistance. Combining various microalgae species allows a hatchery to guarantee the provision of all necessary nutrients that a single species may be deficient in. This mixed-z. It's a close relative of the spirulina derived from saltwater. The cells are spherical, ranging from approximately 4 to 10 µm in diameter, and possess a cup-shaped, pea-green chloroplast that includes a singular pyrenoid for carbon fixation (Chen *et al*., 2024). This freshwater alga asexually reproduces through autospores, where a parent cell splits into several daughter cells when light and nutrient conditions are optimal. Numerous studies indicate that *Chlorella vulgaris* is recognized for its substantial protein levels, digestible enzyme-rich cell wall, and abundant nutritional profile, encompassing all essential

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amino acids, vitamins, minerals, and antioxidants like beta-carotene and chlorophyll. *Chlorella vulgaris* (Chlorella sp.) is defined by its straightforward cultivation, significant productivity, and elevated levels of protein, chlorophyll, lutein, and various vital micronutrients (Buono *et al*., 2014; Choi *et al*., 2021; Hao *et al*., 2021). It can also develop mixotrophically, accumulating lipids and polysaccharides, making it a valuable organism for biofuel generation, wastewater management, and nutraceutical applications. Its flexibility, quick reproduction rate, and effective nutrient uptake render it a suitable model organism for microbiological research and a sustainable resource for biotechnological uses.

MATERIALS AND METHODS

To investigate the importance of the green algae *Chlorella vulgaris* as a supplementary diet in the larval development, survival rate, and growth performance of Penaeus vannamei, both *Chlorella vulgaris* and the diatom *Chaetoceros calcitrans* were obtained from the Central Institute of Brackish water Aquaculture (CIBA) Chennai to serve as starters. This research was carried out in a shrimp hatchery situated 50 km to the south of Chennai city.

Seawater treatment preparation

Previously, the water was channeled into a reservoir. The water was treated with 20 ppm of calcium hypochlorite (CaOCl2) for chlorination. Afterward, the seawater was aerated for six hours. Residual chlorine was measured after 6 hours and neutralized using Sodium Thio-sulphate (Schroeder *et al*., 2010). The water remained untouched for approximately 2 hours to enable the settling of all suspended particles and microorganisms. The seawater was subsequently filtered through a pressure filter, followed by micron filters (1µm, 0.5 µm, and 0.25 µm), and a UV sterilizer prior to use. The physicochemical characteristics of seawater were examined. Salinity measured 30 ppt, pH was 7.9±1, Alkalinity stood at 124 mg/L, hardness was 4252±2 ppm, and total ammonia (NH3) was below 0.04 ppm.

Maintenance of algal strains and media preparation

Two distinct media were created for two types of algae. Chu's No. 10 medium for *Chlorella vulgaris* and Guillard f/2 medium for *Chaetoceros calcitrans*.

Chu's No. 10 media

Table 1. Chu's No. 10 media for *Chlorella vulgaris*

Ingredients	Concentration
Calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$)	40 mg/L
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	25 mg/L
Dipotassium hydrogen phosphate (K_2HPO_4)	5 mg/L
Sodium carbonate (Na_2CO_3)	20 mg/L
Sodium silicate ($Na_2SiO_3 \cdot 9H2O$)	25 mg/L
Iron (II) chloride ($FeCl_2$)	8 mg/L

The medium typically comprises distilled water with added stock solutions of essential nutrients, such as: Calcium nitrate (Ca (NO3)2·4H2 O), Magnesium sulfate (MgSO4·7H2 O), Dipotassium hydrogen phosphate (K2 HPO4), Sodium carbonate (Na2 CO3), Sodium silicate (Na2 SiO3·9H2O), and a trace metal solution containing Iron (II) chloride (Fe Cl2). Every component with different concentrations (Table 1) was dissolved individually in distilled water prior to mixing. The pH was usually modified to 6.4 prior to sterilization. Sterilization was performed through autoclaving at 121°C for 15 minutes, and then it was permitted to cool prior to utilization.

Guillard f/2 (Guillard 1975)

Guillard's F/2 medium is a commonly utilized enriched seawater medium initially developed for the growth of coastal marine algae, particularly diatoms. It is a weakened version (50% strength) of the original "f Medium" created by Guillard and Ryther in 1962. The medium typically comprises filtered natural seawater with the addition of stock solutions of essential nutrients, such as: Nitrate (NaNO3), Phosphate (NaH2PO4·H2O), Silicate (Na2SiO3·9H2O), trace metal solutions, and vitamin solutions. The trace metals consist of iron (FeCl3·6H2O), copper, zinc, cobalt, and manganese, among others, usually complexed with EDTA for enhanced stability. The vitamin mixture includes cyanocobalamin (Vitamin B12), thiamine HCl, and biotin. To make 1 liter of F/2 medium, standard stock amounts that were added to about 950 ml of filtered seawater included 1 ml each of nitrate, phosphate, silicate (optional), and trace metals, along with 0.5 ml of the vitamin solution. The medium underwent autoclaving for sterilization. *Chlorella vulgaris* and *Chaetoceros calcitrans* were obtained from the Central Institute of Brackish water Aquaculture (CIBA) in Chennai and utilized as starters. The starters were adapted to the laboratory environment. The algae *Chlorella vulgaris* and the diatom *Chaetoceros calcitrans* were added to test tubes filled with distinct media for two types of algae. 1 ml of two distinct starters was added to separate test tubes, each containing 9 ml of sterile media, and allowed to develop for 2 days before further dilution. Moreover, these various algae were diluted

and expanded for large-scale production using the same method. The temperature was maintained at 25℃ while the light intensity was set to 2000 lux under indoor conditions (Oostlander *et al*., 2020 and Kanimozhi *et al*., 2021). While the exterior culture remained in natural top-illuminated tanks. The mature algae were observed through the microscope, and cell counting was performed using a hemocytometer.

The experimental group was split into two subgroups. Six glass jars were utilized and labeled as C1, C2, and C3 for the control group and E1, E2, and E3 for the experimental group. All jars were filled with approximately 2 liters of seawater (equipped with an aerator). PCR screened 100 *Penaeus vannamei* nauplii were placed in each jar of both the control and experimental groups. Water conditions and animal well-being were noted prior to the experiment (Table 2). The control group received solely diatom (*Chaetoceros calcitrans*), while the experimental group was given both *Chlorella vulgaris* and *Chaetoceros calcitrans* in alternation. All other parameters and the inclusion of probiotics were carried out correctly for both the control and experimental groups. Algal feed and synthetic feed are supplied to each group twice daily. Daily observations were made regarding water quality, larval feeding behavior, and stage conversion. The experiment was extended for 10 days until all larvae transformed into post larvae. All the information was analyzed statistically.

Table 2. Initial health status of fry and water quality parameters recorded before the experiment.

S.No	Parameters	Control			Experimental		
		C_1	C_2	C_3	E_1	E_2	E_3
1	Pigmentation	Normal	Normal	Normal	Normal	Normal	Normal
2	Appendages	Clear	Clear	Clear	Clear	Clear	Clear
3	Gut	-	-	-	-	-	-
4	Survival %	-	-	-	-	-	-
5	Water salinity ppt	30	30	30	30	30	30
6	Water Tem ° C	31±0.1	31±0.1	31±0.1	31±0.1	31±0.1	31±0.1
7	Water pH	7.9±1	7.9±1	7.9±1	7.9±1	7.9±1	7.9±1
8	Alkalinity mg/L	124	124	124	124	124	124
9	Hardness ppm	4252±2	4252±2	4252±2	4252±2	4252±2	4252±2
10	Total Ammonia NH_3 ppm	≤0.04	≤0.04	≤0.04	≤0.04	≤0.04	≤0.04
11	Water colour	Clear	Clear	Clear	Clear	Clear	Clear
12	Green colony	BDL	BDL	BDL	BDL	BDL	BDL
13	Yellow colony	BDL	BDL	BDL	BDL	BDL	BDL

BDL- Below detectable limit.

RESULTS AND DISCUSSION

The initial health conditions and water quality metrics were documented after the experiment and presented in a table (Table 3). The pigmentation of nauplii observed was deemed normal, and the appendages remained clear throughout the experimental duration in both the control and experimental groups. Although all other parameters, specifically water salinity (30‰), temperature (31±0.1℃), pH (7.9±1), and alkalinity (124 mg/L), were kept constant from the start of the experiment, the water color changed to brown in the control group and greenish in the experimental group. The ammonia level in water was ≤0.04 prior to the experiment, whereas post-experiment it measured ≤0.08, indicating an increase to double the original amount. Guts of the experimental organisms were empty prior to the experiment, while at the conclusion of the experiment, the guts in both groups were observed to be full.

Table 3. Initial health status of fry and water quality parameters recorded after the experiment

S.No.	Parameters	Control			Experimental		
		C_1	C_2	C_3	E_1	E_2	E_3
1	Pigmentation	Normal	Normal	Normal	Normal	Normal	Normal
2	Appendages	Clear	Clear	Clear	Clear	Clear	Clear
3	Gut	Full	Full	Full	Full	Full	Full
4	Survival %	82	69	79	98	92	90
5	Water salinity ppt	30	30	30	30	30	30
6	Water Tem ° C	30±0.1	31±0.1	31±0.1	31±0.1	30±0.1	31±0.1
7	Water pH	7.9±1	7.9±1	7.9±1	8.1±1	8.0±1	8.0±1
8	Alkalinity mg/L	124	124	124	124	124	124

9	Hardness ppm	4252±2	4252±2	4252±2	4252±2	4252±2	4252±2
10	Total Ammonia NH_3 ppm	≤0.08	≤0.08	≤0.09	≤0.04	≤0.04	≤0.04
11	Water colour	Brown	Brown	Brown	Greenish	Greenish	Greenish
12	Green colony	Nil	Nil	Nil	Nil	Nil	Nil
13	Yellow colony	40	52	59	58	102	60

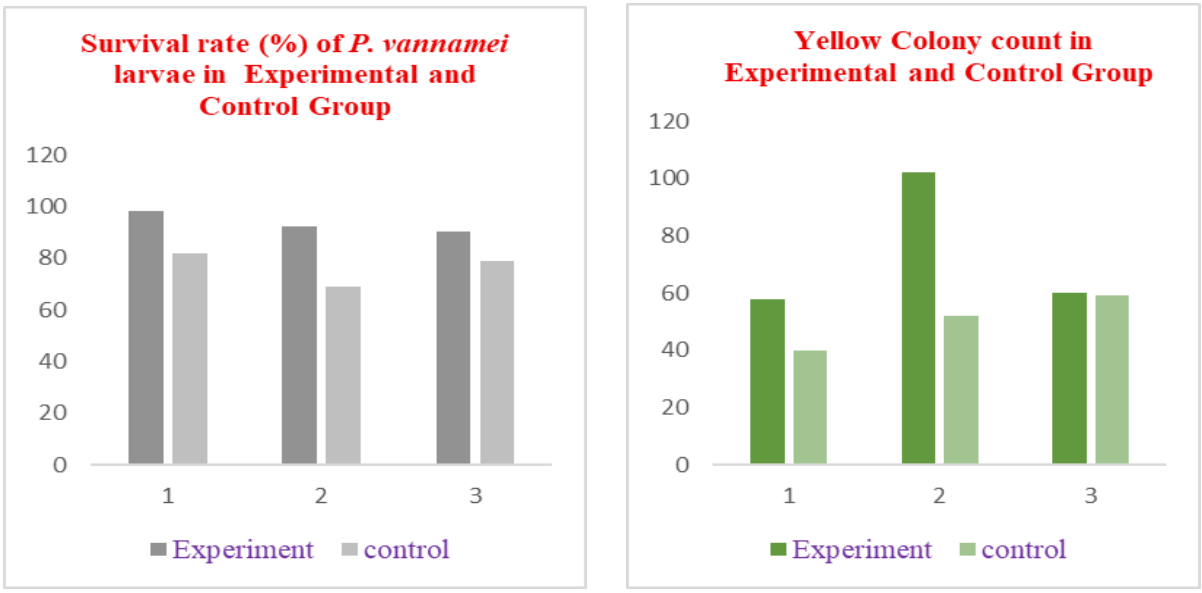

Figure 1. Graph showing survival rate (%) and yellow colony count in Experimental and Control group

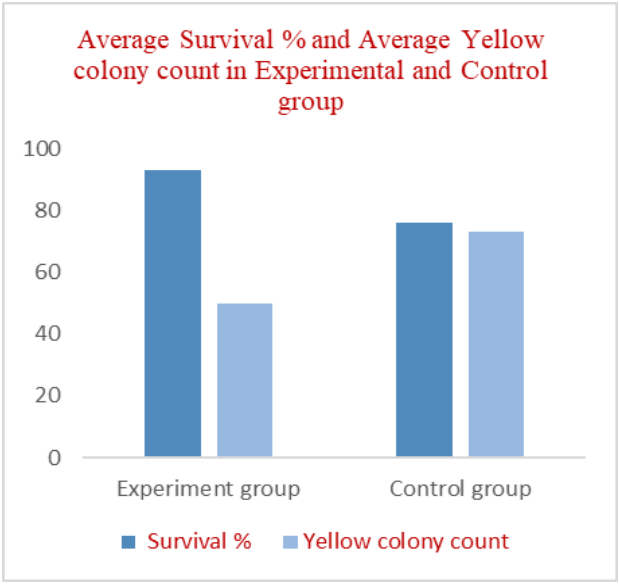

Figure 2. Chart showing Average survival rate (%) and yellow colony count in Experimental and Control group.

The survival rate of post-larval *P. vannamei* and the yellow colony count were noted in all three replicates of both the experimental and control groups (Figure 1). The experimental group's average survival rate of nauplius larvae was 93.3%, notably higher than the control group's survival of 76.6% when fed with diatom (Figure 2). Green colonies were noted prior to beginning the experiment at levels below the detectable threshold. Following the treatment, both the group given the experimental diet and the control group fed with diatom showed no presence of green colonies at the conclusion of the experiment. Yellow colonies were noted in both groups. The control group's average yellow colony count was 151 (50.3%), while the experimental groups was 220 (73.3%). The experimental diet promotes the growth of *P. vannamei* nauplius larvae into post-larvae and effectively enhances their survival rate. *Vannamei* nauplii that were given diatoms and supplementary feed exhibited markedly reduced survival rates in comparison to those that received the experimental diet. The findings of the experiment align with previous findings by Li *et al*. (2022), who noted that effectively substituting 20% of dietary fish meal with *Chlorella sorokiniana* meal greatly enhanced the growth performance of *L. vannamei* and lowered mortality rates (Cui *et al*., 2019). Control organisms that received diatoms in combination with feed supplements demonstrated a larval survival rate of 76.6%. This outcome aligns with the findings of Godoy *et al*. (2011), who showed that adding diatoms during the nursery phase of *L. vannamei* enhanced shrimp survival.

Microalgae provide a nutrient-dense food source for cultured fish, crustaceans, and their juvenile stages (Conceiao *et al*., 2010; Courtois de Vicose *et al*., 2012). Microalgae products are safe for consumption, contain suitable proteins/fats, functional carbohydrates, and various biological indicators such as bioactive peptides, antimicrobial properties, unknown growth factors, and can be cultured easily, making them regarded as valuable feed ingredients in aquaculture (Ahmad *et al*., 2020; Bito *et al*., 2020). A prior study indicated that incorporating *Chlorella vulgaris* into the feed can significantly enhance both the specific growth rate and survival rate of *Litopenaeus vannamei* (Pakravan *et al*., 2017). The results of this study emphasized the significance of using *Chlorella vulgaris* together with diatom (*Chaetoceros calcitrans*) as food for larvae, along with feed additives, for the development and survival of *P. vannamei* nauplius larvae through to post-larval stages. In summary, including *Chlorella vulgaris* in the diet significantly improved the growth and survival rate of *P. vannamei*. In summary, this research showed that diatom in shrimp feed can be substituted with *C. vulgaris* in the diet of nauplius larvae of *P. vannamei* without negatively impacting shrimp performance.

CONCLUSION

Findings indicated that *Penaeus vannamei* shrimp larvae nourished with the microalga *Chlorella vulgaris* combined with diatom (*Chaetoceros calcitrans*) showed improved survival rates and larval change from nauplius to post larvae compared to those in the control group, which was exclusively fed diatom. Shrimp nourished with *Chlorella vulgaris* surpassed those nourished with diatom.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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